Modulation of regulatory T cell suppression in tumors through OX40

Thesis

Modulation of regulatory T cell suppression in tumors through OX40

by

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ABSTRACT

Tumor cells develop numerous mechanisms to escape from the control exerted by the immune system. One of these strategies is the accumulation of regulatory T cells (Treg) within the tumor, which keep effector T cells (Teff) and dendritic cells (DC) in an inactive state. An efficient approach to overcome the inhibitory potential of Treg focuses on OX40, a costimulatory molecule constitutively expressed by Treg and induced in activated Teff. The treatment of mouse transplantable tumor models with the mAb OX86, the agonist of OX40, induces tumor rejection by acting on both these T cell subsets. In this study we investigated the fine cellular mechanisms at the basis of this process, dissecting the effects of OX86 on Treg and on $CD4^{+}Foxp3^{-}CD44^{high}CD62L^{low}OX40^{+}$ effector memory T cells (Tem), which represent the most abundant Teff subset in the tumor. Upon OX40 stimulation, Treg are "contra-suppressed" and down-modulate the expression of the transcription factor interferon regulatory factor 1 (IRF1), thus reducing the secretion of IL-10. Conversely OX86 provides activating stimuli to Tem, which up-regulate CD40L and in turn promote the maturation of DC. OX86 shifts the tumoral milieu from tolerogenic to immunogenic, favoring the activation and migration of DC from the tumor to the draining lymph node (dLN) and the subsequent new CTL induction.

The relevance of OX40 in Treg biology goes beyond the modulation of their suppressive abilities. OX40 increases the sensitivity of Treg to IL-2, facilitating the phosphorylation of STAT5 through high level of the mir155 and low level of SOCS1. The overexpression of miR155 endowed Treg of higher suppressive functions, further enhancing tumor growth.

These data clearly remark the key roles exerted by OX40 in influencing Treg and Teff behavior. Understanding how to manipulate OX40 signaling will provide great advantage in the development of efficient therapy for both tumors and autoimmune diseases.

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ABBREVIATIONS

Ad: adenovirus

Ag: antigen

AHR: airway hyper responsiveness

AICD: activation-induced cell death

APC: allophycocyanin

APC: antigen presenting cells

BM: bone marrow

BM-DC: bone marrow-derived dendritic cell

BS: binding site

cAMP: cyclic adenosine monophosphate

CD: cluster of differentiation

CTL: cytotoxic T lymphocytes

CTLA-4: cytotoxic T lymphocytes-associated antigen 4

CTX: cyclophosphamide

DAMPs: damage-associated molecular pattern

DC: dendritic cells

DN: double negative

DP: double positive

dLN: draining lymph node

ds: double-strand

EAE: experimental autoimmune encephalomyelitis

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FC: fold change

FITC: fluorescein isothiocyanate

Foxp3: factor forkhead box P3

GC: germinal center

GFP: green fluorescent protein

GITR: glucocorticoid-induced TNF receptor family-related gene

GM-CSF: granulocytes-macrophages colony stimulating factors

GVHD: graft versus host disease

GZ: granzyme

HCC: hepatocellular carcinoma

i.p: intraperitoneal

IPEX: immunedysregulation polyendocrinopathy enteropathy X-linked syndrome

i.t: intratumor

iTreg: induced regulatory T cells

IBD: inflammatory bowel disease

ICS: intra cellular staining

IDO: indoleamine 2,3-dioxygenase

IFN: interferon

IL-: interleukin

IONO: Ionomycin Calcium Salt

Kb: kilobase

KO: knock-out

LAP: latency associated peptide

LPS: lipopolysaccharides

mAb: monoclonal antibody

MC: mast cell

MDSC: myeloid derived suppressor cells

MFI: mean fluorescent intensity

MHC: major histocompatibility complex

miRNA: microRNA

mm: millimeters

mRNA: messenger RNA

NF-kB: nuclear factor-kappaB

NK: natural killer cells

NKT: natural killer T cells

NOD: non-obese diabetic mouse

nt: nucleotide

nTreg: natural regulatory T cells

ORF: open reading frame

PBS: phosphate buffered saline

pDC: plasmacytoid dendritic cells

PE: R-Phycoerythrin

PMA: Phorbol Myristate Acetate

PBMC: peripheral blood mononuclear cell

RA: rheumatoid arthritis

RISC: RNA-induced silencing complex

Stat: signal transducer and activator of transcription

s.c: subcutaneous

SP: single positive

ss: single-strand

T1D: type-1 diabetes

Tact: T recently activated

TAM: tumor-associated macrophages

TB: tumor-bearing

Tcm: T central memory

TCR: T cell receptor

Teff: effector T cells

Tem: T effector memory

TF: transcriptor factor

TF: tumor free

Tfh: T-follicular helper

Tg: transgenic

TGFβ: transforming growth factor beta

Th: T helper

TIDC: tumor-infiltrating dendritic cells

TIL: tumor-infiltrating lymphocytes

Tn: T naïve

TNF: tumor necrosis factor

TNFR: tumor necrosis factor receptor

TRAIL: tumor necrosis factor-related apoptosis inducing ligand

Treg: regulatory T cells

TSLP: thymic stromal lymphopoietin

UTR: untranslated region

VEGF: vascular endothelial growth factor

wt: wild type

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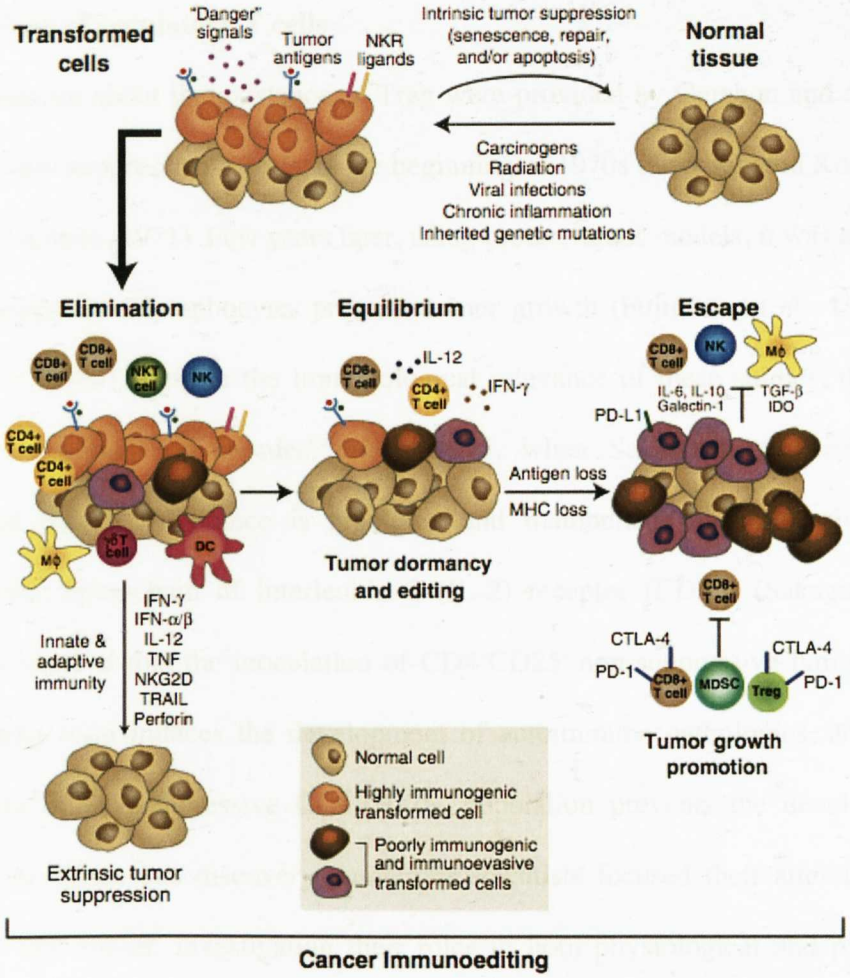
1 INTRODUCTION

1.1 The cancer immunoediting theory

At the beginning of the 20th century Paul Ehrlich was the first to theorize that the cells of the immune system could control and prevent the development of tumors (Ehrlich, 1909). At that time however the poor knowledge of the immune system did not allow this theory to be adequately investigated. Only 50 years later, when tumor-associated antigens were discovered (Old and Boyse, 1964), the interaction between immune cells and transformed cells, renamed "immunosurveillance", was reconsidered and subsequently validated (Smyth and Trapani, 2001). The immunosurveillance theory took advantage from the generation of genetically modified mice and the development of specific monoclonal antibodies (mAbs) that block cytokines and molecules related to immune system functions. New studies on the immune system put in evidence that the interactions between tumors and immune cells were more complex than those supposed in the immunosurveillance theory. In particular it was discovered that cells of both innate and adaptive immune system not only prevent tumor onset, but also modify the immunogenicity of tumor cells (Dunn et al., 2002; Shankaran et al., 2001). These observations rendered the immunosurveillance concept insufficient to explain the relationship between tumor and immune system and induced the use of the "cancer immunoediting" term to better define the double effect of the immune system on tumor. The cancer immunoediting process consists of three different phases: elimination, equilibrium, escape (Dunn et al., 2004). The elimination phase corresponds to the initial concept of immunosurveillance. In this stage cells of both innate (NK cells and macrophages) and adaptive ($CD4^+$ and $CD8^+$ lymphocytes) immune system are able to recognize and eliminate transformed cells. The specific mechanisms that guide this process are not fully understood, however it seems that type I interferons (IFNs,) damage-associated molecular pattern molecules (DAMPs) and

stress ligands could promote the activation of immune cells, which in turn work as an extrinsic tumor suppressor mechanism. When neoplastic clones survive the check exerted by the immune system, they enter in the equilibrium phase. In this phase, which is considered the longest one of the all immunoediting process, immune system keeps latent tumor cells under control, preventing the outgrowth of clinically evident tumors. Last is the escape stage, when tumor cells get the ability to overcome the block exerted by immune cells and initiate the evident pathology. The escape of tumor cells may be due to different mechanisms, which include reduced immunogenicity (low expression level of MHC class I and loss of antigen expression), acquired resistance to the cytotoxic functions of immune cells and accumulation in the tumor microenvironment of immunosuppressive cells, like regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) (Dunn et al., 2004; Schreiber et al., 2011). Both Treg and MDSC act in order to inhibit effector cell functions, thus preventing the development of an efficient immune response against tumor cells. Many studies, both in mouse and in human, have reported an increased accumulation of Treg both in tumor mass and peripheral blood in cancer patients; for instance, an augmented proportion of Treg was found in the peripheral blood of patients with pancreatic tumor (Liyanage et al., 2006), breast cancer (Liyanage et al., 2002), ovarian cancer (Woo et al., 2001), colorectal cancer (Deng et al., 2010), lymphoma (Marshall et al., 2004) and melanoma (Correll et al., 2010). The awareness that the immune system has a double role in regulating tumor onset, acting both as an extrinsic tumor suppressor and as a tumor sculpting player, has favoured the idea that modulating immune cell abilities in the right manner will allow the development of efficient anti-tumor therapy. In this regard, discovering how to inhibit suppressive cells (Treg) and stimulate effector cells (Teff) will be very helpful for the establishment of powerful cancer immunotherapies.



(From Schreiber R.D., et al, 2011)

Figure 1.1: the cancer immunoediting process. The first phase of the immunoediting process is the elimination. During this stage immune cells are able to recognize and eliminate transformed cells. The subsequent step is the equilibrium phase, when immune cells fail in eliminating all tumor clones, but just keep them under control preventing the onset of clinically evident pathology. Finally tumor cells acquire the ability to escape from the immunological pressure, thank to the development of a series of immunosuppressive strategies like antigen loss, MHC down-regulation, accumulation of Treg and recruitment of MDSC in tumor microenvironment.

1.2 The biology of regulatory T cells

The first evidence about the existence of Treg were provided by Gershon and colleagues, who called them suppressive T cells, at the beginning of 1970s (Gershon and Kondo, 1970; Gershon and Kondo, 1971). Few years later, using mouse tumor models, it was also proven that these suppressive lymphocytes promote tumor growth (Fujimoto et al., 1975; North and Bursuker, 1984). Despite the immunological relevance of these studies, the field of Treg was abandoned for decades, up to 1995, when Sakaguchi and collaborators demonstrated that self-tolerance is regulated and maintained by suppressive T cells expressing the alpha-chain of interleukin 2 (IL-2) receptor (CD25) (Sakaguchi et al., 1995). They showed that the inoculation of $CD4^+CD25^-$ non-suppressive lymphocytes in BALB/c nu/nu mice induces the development of autoimmune pathologies, and that the reconstitution of the suppressive $CD4^+CD25^+$ population prevents the development of those diseases. Since this discovery, numerous scientists focused their attention on this particular T cell subset, investigating their roles in both physiological and pathological conditions.

IL-2Rα (CD25), with IL-2Rβ (CD122) and γc (CD132), constitute the IL-2 receptor (IL-2R) (Malek and Castro, 2010). The assemblage of IL-2R is a cascade process: at the beginning IL-2 binds to the CD25 subunit and this interaction causes a conformational modification in IL-2 structure, which allows the binding of CD122 to IL-2. Finally the γc subunit is included in the structure and renders it more stable (Malek and Castro, 2010). IL-2 signaling is crucial for Treg development and consequently for peripheral homeostasis; indeed mice with impaired IL-2/IL2R axis are affected by lethal autoimmune diseases mainly due to alteration in regulatory lymphocytes (Malek, 2003; Malek and Castro, 2010; Malek et al., 2002). The intracellular signaling of IL-2 is primarily mediated by signal transducer and activator of transcription 5 (Stat5) (Burchill et al., 2007).

It was also demonstrated that Treg constitutively express basal level of CD40L, such to stimulate DC, via CD40, to produce sufficient amount of IL-2 to assure their survival. The relevance of CD40/CD40L axis in Treg biology was clearly observed in CD40 KO mice, in which Treg have lower proliferative and survival potential (Guiducci et al., 2005a).

At the beginning of the 21st century, the discovery that the transcription factor forkhead box P3 (Foxp3), on X chromosome, was mutated both in scurfy mice and in human with immunedysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (Brunkow et al., 2001; Wildin et al., 2001) led to the idea that this transcriptor factor could be involved in Treg development. In fact two years later Foxp3 was identified as the master regulator for Treg differentiation and functions (Fontenot et al., 2003; Fontenot et al., 2005b; Hori et al., 2003). It was demonstrated not only that in absence of Foxp3 the development of Treg is impaired, but also that the ectopic expression of Foxp3 in $CD4^+CD25^-$ naïve T cells endow them with suppressive functions (Fontenot et al., 2003; Hori et al., 2003). Thanks to the generation of a knock-in Foxp3-GFP mice, in which the complete eGFP sequence was inserted in the first exon of the Foxp3 gene (Fontenot et al., 2005b), it was possible to specifically identify which cell subset expresses this transcription factor. It was found that Foxp3 is mainly expressed (>99,8%) by $TCR\beta^+$ T cells, both in thymus and periphery, while macrophages, DC cells, NKT cells, NK cells, B lymphocytes and non hematopoietic cells are deficient in Foxp3 (Fontenot et al., 2005b). Foxp3 expression was confirmed in both $CD25^+$ and $CD25^-CD4^+$ T cells, whose suppressive abilities were confirmed with different functional assays. Thank to these studies Foxp3 was considered the most specific Treg marker, since CD25, and other surface molecules constitutively expressed by Treg, like glucocorticoid-induced TNF receptor family-related gene (GITR), cytotoxic T lymphocytes-associated antigen 4

(CTLA-4) and OX40, are up-regulated also by activated Teff (Fontenot et al., 2005b; Shimizu et al., 2002; Takahashi et al., 2000; Takeda et al., 2004).

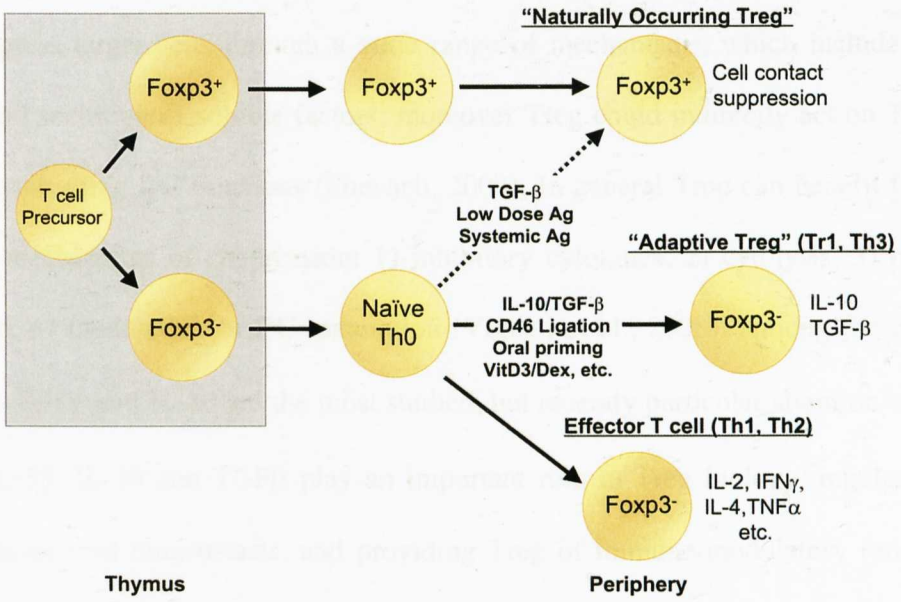
1.2.1 Regulatory T cell development

Treg could differentiate both in the thymus, from early common precursors, and in periphery, from naïve $CD4^+Foxp3^-$ T cells in response to different immunosuppressive stimuli (Feuerer et al., 2009; Mailloux and Young, 2010); the former are named natural Treg (nTreg), the latter induced (or adaptive) Treg (iTreg). In the thymus, Foxp3 expression is predominantly found at the stage of $CD4^+$ single positive (SP) cells, although a low percentage of cells expressing Foxp3 is found in the $CD8^+$ SP, $CD4^+CD8^+$ double positive (DP) and $CD4^-CD8^-$ double negative (DN) stages of thymic development (Fontenot et al., 2005b). Although the precise signals that guide nTreg differentiation, and therefore Foxp3 expression, are not still well understood, a crucial step in this process seems to be the strong interaction between TCR and MHC/self-antigen presented by DC (Cozzo Picca et al., 2011; Jordan et al., 2001; Mailloux and Young, 2010). Indeed, in mice lacking both MHC class-I and MHC class-II, Foxp3 expression is lost (Fontenot et al., 2005b). Also IL-2 is a critical player in nTreg differentiation process (Cheng et al., 2011; Feuerer et al., 2009). It was demonstrated that mice with alteration in the IL-2/IL-2R pathway (Fontenot et al., 2005a; Malek et al., 2002), or in IL-2 signals transducers, like Stat5 and Jak3 (Sakaguchi et al., 2008), or treated with anti-IL-2 antibody (Bayer et al., 2005), have few Treg, both in thymus and periphery, compared to wt mice. In addition these Treg express Foxp3 at lower level than wt Treg, resembling immature and non-suppressive regulatory T cells (Tran et al., 2007). In 2008 Hsieh and Ferrar, in two distinct studies, described a two-step model to explain nTreg development in the thymus depending on TCR and IL-2 signals (Burchill et al., 2008; Lio and Hsieh, 2008). They

proposed that nTreg precursors ($CD4^+CD25^{high}Foxp3^-TCR^+$), after receiving strong stimulation via TCR, further up-regulate CD25, thus becoming more sensitive and responsive to IL-2. This enhanced IL-2 responsiveness at last promotes Foxp3 expression (Burchill et al., 2008; Lio and Hsieh, 2008).

In addition to the stimuli provided by TCR and IL-2, other molecules were indicated as relevant in the nTreg differentiation process. Among these the costimulatory molecule CD28 (Salomon et al., 2000; Tai et al., 2005) and the transcription factor NF-kB (Guckel et al., 2011; Medoff et al., 2009) were demonstrated to be necessary for nTreg differentiation.

Unlikely nTreg, iTreg differentiate in periphery from naïve T cells in response to different stimuli. iTreg include different subsets of regulatory cells: Tr1, Th3 and Treg indistinguishable from nTreg (Wing et al., 2006). When naïve T cells are in presence of high amount of IL-10 they acquire a suppressive phenotype and are defined Tr1 cells. These cells produce abundant IL-10, but do not secrete TGFβ (Groux et al., 1997). On the contrary Th3 cells are induced by and produce TGFβ (Chen et al., 1994). Both Tr1 and Th3 cells, even if endowed of suppressive functions, do not express Foxp3. Naïve lymphocytes can also differentiate in iTreg indistinguishable from nTreg when are in presence of TGFβ and insufficient antigen stimulation by immature/tolerogenic APC (Wing et al., 2006)



(From Wing K., et al, 2006)

Figure 1.2: Treg development. Regulatory T cells differentiate from both common thymic precursors and from naïve $CD4^+$ T cells in periphery. Thymus-derived Treg (nTreg) develop in response to strong TCR stimulation and IL-2 signal. iTreg differentiate in periphery from $CD4^+Foxp3^-$ lymphocytes in response to different stimuli (IL-10, TGFβ, CD46 ligation). iTreg are classified in two main subsets: Tr1 which preferentially suppress in an IL-10 fashion manner and Th3, which secrete high amount of TGFβ. In addition to iTreg, other Treg, indistinguible from nTreg, differentiate in periphery from naïve T cells (Th0), in response to low dose of TGFβ and inefficient Ag presentation. These Treg and nTreg suppress target cells in a cell-cell contact manner.

1.2.2 Regulatory T cell suppressive mechanisms

Treg suppress target cells through a wide range of mechanisms, which include cell-cell contact and secretion of soluble factors; moreover Treg could indirectly act on T effector cells by controlling DC functions (Shevach, 2009). In general Treg can benefit from four different mechanisms of suppression: 1) inhibitory cytokines, 2) cytolysis, 3) metabolic disruption, 4) modulation of DC maturation (Vignali et al., 2008). Among the inhibitory cytokines TGFβ and IL-10 are the most studied, but recently particular attention was given also to IL-35. IL-10 and TGFβ play an important role in Treg biology, regulating their differentiation and homeostasis, and providing Treg of immune-modulatory functions. It was demonstrated that these cytokines are required to cure a wide range of diseases, including inflammatory bowel disease (IBD), colitis, lung allergy and airway inflammation (Chaudhry et al., 2011; D'Alessio et al., 2009; Whitehead et al., 2011). Treg could also suppress target cells with a form of membrane-bound TGFβ in a cell-cell contact manner (Nakamura et al., 2001). IL-35 was recently described as a critical cytokine for the fully suppressive function of Treg. IL-35 is a heterodimeric cytokine formed by Epstein-Barr-virus-induced gene 3 (Ebi3) and IL-12α (p35), and is highly expressed by Treg but not by resting effector T cells. It was shown that IL-35 is required to cure IBD (Collison et al., 2007), prevent collagen-induced arthritis via IL-10 (Kochetkova et al., 2010), and block IL-17-dependent airway hyperresponsiveness (AHR) (Whitehead et al., 2011).

The second mechanism of Treg suppression is cytolysis. Thank to experiments conducted in granzyme B deficient mice (GZ-B$^{-/-}$), it was demonstrated that this enzyme is critical for Treg functions (Gondek et al., 2005), and that Treg promote tumor growth by secreting high level of granzyme B and perforin, thus reducing anti-tumor CTL response (Gondek et al., 2005). Galectin-1 (Garin et al., 2007) and TRAIL (tumor necrosis factor-related apoptosis inducing ligand) (Ren et al., 2007) have been recently identified as important

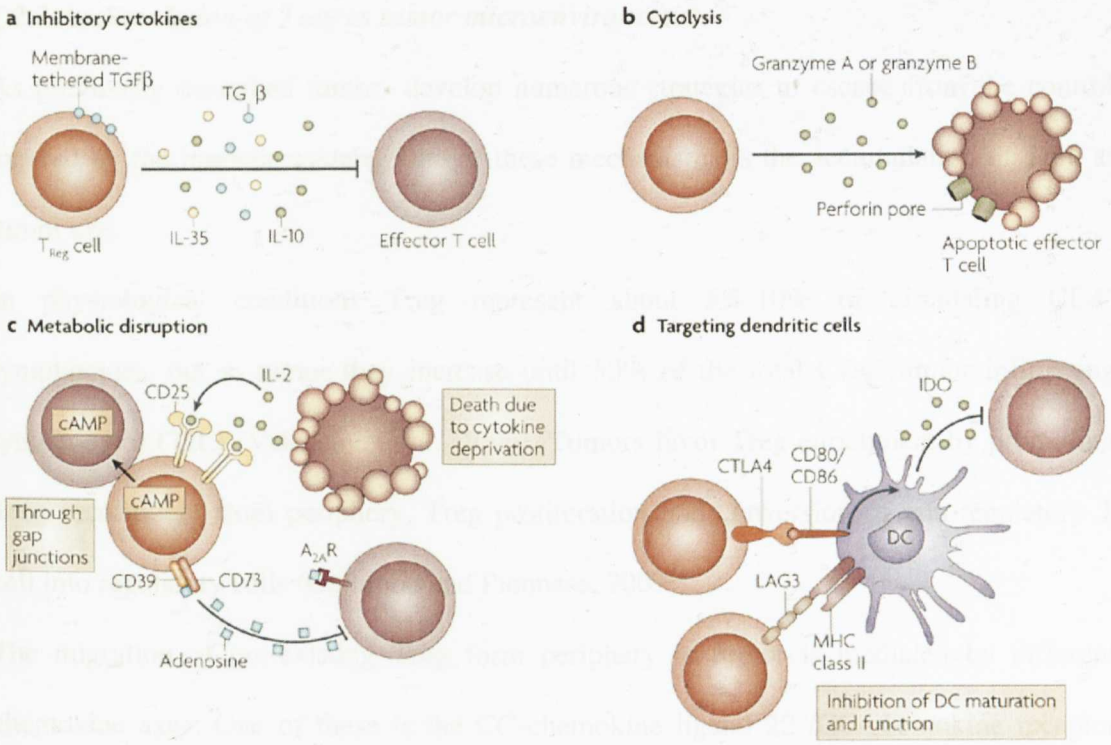
mediators of cell death. Galectin-1 is a β-galactoside binding protein, which interacts with CD45, CD43 and CD7. Upon TCR engagement Treg up-regulate its expression and reduce proliferation, survival and cytokine secretion by effector T cells (Shevach, 2009). Ren and collaborators in 2007 demonstrated that TRAIL, binding to DR5, induces $CD4^+Foxp3^-$ T cells apoptosis both *in vitro* and *in vivo*, and that the blockage of TRAIL/DR5 interaction significantly reduces Treg cytotoxicity and the survival of mice after allogenic skin graft (Ren et al., 2007).

Another Treg suppressive strategy consists in the deprivation of cytokines necessary for effector T cells survival, like IL-2. It is well established that IL-2 is a crucial cytokine for Treg development and survival and that Treg constitutively express CD25. However this molecule is also important for effector T cell proliferation and survival upon activation. During inflammation Treg compete for IL-2 with effector T cells, thus consuming it and inducing effector T cells starvation and apoptosis (Pandiyan et al., 2007). Metabolic disruption is also induced by the generation of pericellular adenosine by the two enzymes CD39 and CD73, which are expressed on Treg surface (Deaglio et al., 2007). CD39 and CD73 are involved in the modulation of immune responses in pathological conditions; for instance $CD73^{-/-}$ mice develop a stronger anti-tumor immune response compared to CD73 sufficient mice (Stagg et al., 2011) and patients with multiple sclerosis have a reduced number of $CD4^+Foxp3^+CD39^+$ regulatory cells than healthy control patients (Fletcher et al., 2009). Another adenosine molecule, the intracellular cyclic adenosine monophosphate (cAMP), endowed of strong immune-suppressive function, is transferred from Treg to effector T cells though gap-junctions and blocks Teff proliferation and IL-2 synthesis (Bopp et al., 2007).

Finally Treg can also affect Teff activation indirectly, by modulating DC activation. DC have the crucial role of providing costimulatory signals to T cells allowing their fully

activation (Matzinger, 2002). Therefore Treg, hampering DC maturation, also block Teff activation. In this regard a strategy developed by Treg is their constitutive expression of the cytotoxic T lymphocytes-associated antigen 4 (CTLA-4) (Read et al., 2000). CTLA-4 down-modulates CD80 and CD86 expression level on DC, thus impairing Teff activation via CD28 (Wing et al., 2008). In addition, upon CTLA-4/CD80-CD86 interaction, DC are induced to express the indoleamine 2,3-dioxygenase (IDO) enzyme, which promotes the catabolism of tryptophan into the pro-apoptotic metabolite kynurenine (Fallarino et al., 2003). DC maturation and function are also impaired by lymphocyte activation gene 3 (LAG-3 or CD223), a homolog of CD4, that binds to MHC class II, thus reducing the ability of DC to present antigens to effector lymphocytes (Huang et al., 2004; Liang et al., 2008). Recently it was demonstrated that the transmembrane protein neuropilin (nrp-1) is required for long Treg-DC interaction and increases Treg suppressive functions (Sarris et al., 2008; Sarris and Betz, 2011).

Treg are capable of numerous and dissimilar suppressive mechanisms; understanding which one is preferentially used in different anatomical districts, both in physiological and pathological conditions, will provide great advantage for the development of efficient therapies against tumors and autoimmune diseases.



(From Vignali D., et al, 2008)

Figure 1.3: Treg suppressive mechanisms. Regulatory T cells use different strategies to inhibit target cells. a) Treg release inhibitory cytokines, like IL-10, TGFβ and IL-35, to suppress responder cells. b) Treg promote apoptosis of target cells by secreting granzyme A and B and perforin. c) Regulatory T cells also suppress Teff by limiting the availability of cytokines, like IL-2, necessary for Teff functions. Moreover Treg could produce adenosine and cAMP, endowed of strong immunosuppressive potential. d) Treg impair the maturation and activation of antigen presenting cells, in particular of DC. Treg express LAG3, an analog of CD4, which binds to MHC class II and blocks the interaction between MHC class II and CD4, thus preventing Teff activation. Treg, through the surface molecule CTLA4, reduce the expression of the costimulatory molecules CD80/86 on DC surface and also induce DC to express IDO, which in turn promotes the generation of soluble tryptophan-derived pro-apoptotic metabolites.

1.2.3 Accumulation of Treg in tumor microenvironment

As previously described tumors develop numerous strategies to escape from the control exerted by the immune system. One of these mechanisms is the accumulation of Treg at tumor site.

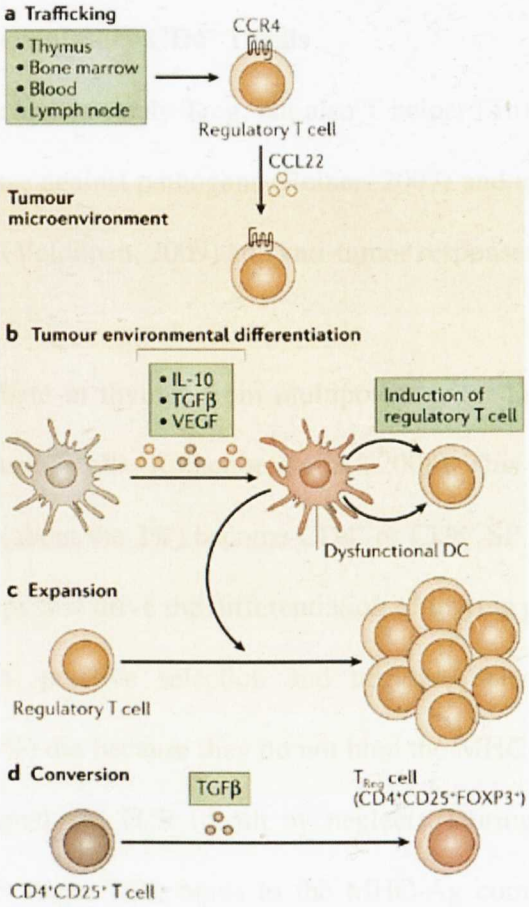
In physiological conditions Treg represent about 5%-10% of circulating $CD4^+$ lymphocytes, but in tumor they increase until 50% of the total $CD4^+$ tumor infiltrating lymphocytes (TIL) (Valzasina et al., 2006). Tumors favor Treg enrichment by promoting Treg recruitment from periphery, Treg proliferation and conversion of non-regulatory T cell into regulatory cells (Colombo and Piconese, 2007).

The migration of pre-existing Treg form periphery to tumor is mediated by different chemokine axes. One of these is the CC-chemokine ligand 22 /CC-chemokine receptor type 4 (CCL22/CCR4) pathways. Transformed cells or immune cells re-educated in tumor microenvironment secrete CCL22, thus enhancing $CCR4^+$ Treg recruitment from different anatomical districts (Curiel et al., 2004; Gobert et al., 2009; Mailloux et al., 2010). Recently, using a model of murine breast cancer, it was demonstrated that a CCR4 blocking antibody, in combination with vaccination, facilitates the development of specific anti-tumor CTL response (Pere et al., 2011). Also the CCR6/CCL20 chemokine pathway is important for the migration of circulating Treg to tumor. It was demonstrated that in patients with hepatocellular carcinoma (HCC) Treg recruitment in tumor mass is tightly dependent on the CCR6/CCL20 axis (Chen et al., 2011).

In tumor microenvironment the high amount of immunosuppressive cytokines (TGFβ, IL-10, VEGF) promotes the in situ proliferation of Treg and the conversion of naïve/effector cells into regulatory cells (Valzasina et al., 2006). For instance TGFβ secreted by tumor-infiltrating DC is required for $CCR6^+$ Treg proliferation (Xu et al., 2011). Tumor cells are also able to convert DC into immature myeloid DC, which promote Treg proliferation via

TGFβ (Ghiringhelli et al., 2005). It was shown that tumor-derived TGFβ also guides the conversion of naïve T cells into Treg (Chen et al., 2003), and this process was confirmed both in murine and human tumors (Lu et al., 2011; Moo-Young et al., 2009). IL-10 is a strong immunosuppressive cytokine and is produced in large amounts in tumors (Wilke et al., 2011). IL-10 reduces MHC, CD80 and CD86 expression on DC (Commeren et al., 2003), thus hampering DC activation and indirectly promoting Treg differentiation. Moreover IL-10 can directly inhibit Teff functions (Joss et al., 2000), thus in turn enhancing Treg inhibitory mechanism. In such immunosuppressive cytokine milieu, tumor-associated APC are kept in an immature state and their ability to present antigens to lymphocytes is impaired. These defective APC promote the conversion of non-regulatory cells into Treg (Kretschmer et al., 2005).

IDO, an enzyme involved in tryptophan catabolism, plays an important role in regulating Treg stability (Sharma et al., 2009) and conversion of naïve T cell in Treg (Fallarino et al., 2006). In tumor-bearing mice, IDO^+ plasmacytoid DC (pDC) attribute to Treg strong suppressive functions (Sharma et al., 2007). Neoplastic cells themselves can express IDO and enhance the expansion of Treg subset by the conversion of non-regulatory T cells (Curti et al., 2007). Moreover, increased expression of IDO in tumor correlates not only with higher percentage of tumor-associated Treg, but also with augmented metastatization (Yu et al., 2011).



(From Zou W., 2006)

Figure 1.4: Treg accumulation at tumor site. Treg accumulation at tumor site is due to different signals, which promote a) Treg recruitment from different anatomical districts (thymus, bone marrow, blood and lymph node) in a chemokine dependent manner, b-c) differentiation and proliferation of Treg induced by dysfunctional DC and d) conversion of Teff into Treg caused by high concentration of suppressive cytokines, like TGFβ, in the tumor microenvironment (Zou, 2006).

1.3 The biology of non-regulatory CD4$^+$ T cells

CD4$^+$ T lymphocytes enclose not only Treg, but also T helper (Th) cells, which coordinate adaptive immune response against pathogens (Reiner, 2007) and are additionally involved in autoimmune diseases (Veldhoen, 2009) and anti-tumor responses (Schreiber et al., 2011; Shankaran et al., 2001).

T lymphocytes differentiate in thymus from multipotent progenitors into CD4$^+$ or CD8$^+$ single positive (SP) mature T cells (Rothenberg et al., 2008). This process is very complex and just few precursors (about the 2%) become CD4$^+$ or CD8$^+$ SP functional lymphocytes. There are three main steps that drive the differentiation of thymic precursors into mature T cells: death by neglect, positive selection and negative selection. The majority of CD4$^+$CD8$^+$ DP cells (98%) die because they do not bind the MHC-Ag complex and do not receive any survival signal via TCR (death by neglect). During the positive selection, which occurs at the DP stage, TCR binds to the MHC-Ag complex on thymic cortical epithelial cells. When the TCR/MHC-Ag interaction correctly happens, T cells receive survival signals; on the contrary those lymphocytes unable to bind MHC die for apoptosis. The fate of DP lymphocytes to become CD4$^+$ or CD8$^+$ SP cells is also decided in the course of the positive selection; indeed, if the TCR properly interacts with the MHC class II, T cells maintain the expression of CD4 and lose CD8; on the contrary, if the correct interaction happens between TCR and MHC class I, the DP cells become CD8$^+$ SP lymphocytes. Lymphocytes that have overcome the positive selection are subjected to the negative selection. In this phase T cells bind to the MHC-Ag complex presented by APC, and if the interaction is too strong T cells receive pro-apoptotic signals and die. The negative selection is a crucial step for assuring immunological self-tolerance, but not all auto-reactive clones are eliminated during thymic selection, for this reason Treg abilities are required to control these cells in periphery. Once mature, CD4$^+$ T cells, which are in

the naïve state (Tn), migrate from thymus to periphery. Tn cells become active upon TCR engagement and adequate costimulatory signals provided by APC. According to the different signals they receive from the microenvironment during activation, Tn cells may differentiate into at least four classes of activated helper T cells: Th1, Th2, Th17 and Th9 lymphocytes (Zhu and Paul, 2010).

1.3.1 Th subsets

A network of cytokines and transcription factors (TF) regulates the differentiation of circulating Tn cells in one of the four Th subsets, upon the encounter with the antigen and the subsequent activation (Zhu and Paul, 2010). Each group is distinguishable from the other ones based on the profile of the secreted cytokines and the expression of specific TF. The first Th subsets identified were the Th1 and Th2 ones. Th1 cells are important for the development of immune responses against intracellular pathogens, secrete high amount of interferon-γ (IFN-γ) and specifically express the TF T-bet. T-bet not only is expressed by already differentiated Th1 lymphocytes, but also, together with IL-12, is indispensable for the conversion of Tn into Th1 (Zhu and Paul, 2010). Th2 are specialized in the production of IL-4, IL-5, IL-9, IL-10, IL-13 and IL-15 and their differentiation is driven by the TF Gata3. Th2 lymphocytes fight against extracellular parasites. The TF RORγt is the master regulator of Th17 differentiation. These cells secrete IL-17 (A-F) and IL-22 and are activated against fungi and extracellular bacteria. Th9 cells belong to the most recently identified Th subset. These cells differentiate in the presence of IL-4 and TGFβ and the molecule PU.1 seems to be the key TF for their development (Goswami et al., 2011; Perumal and Kaplan, 2011).

Another recently described lineage of $CD4^+$ lymphocytes is that of T-follicular helper cells (Tfh). These cells are localized in the B-cell follicle and the interaction between Tfh and B

cells is crucial for B cell activation, differentiation of plasma cells and memory B cell, and germinal center (GC) formation. This cross-talk is also important for Tfh biology (Nutt and Tarlinton, 2011). Tfh are characterized by the expression of the TF Bcl6, and also express CCR7, C-X-C chemokine receptor type 5 (CXCR5), programmed death 1 (Pd-1), inducible T-cell co-stimulator (ICOS) and secrete IL-21 (Crotty, 2011; Nutt and Tarlinton, 2011).

1.3.2 $CD4^+$ T cell activation

$CD4^+$ T cells activation requires not only TCR engagement by Ag/MHC class II complex, but also second activator signals provided by costimulatory molecules expressed by APC. The relevance of these signals is proven by the fact that T cells stimulated via TCR, but lacking costimulation, die (Matzinger, 2002). Costimulatory molecules can be classified according to their molecular structure or their specific functions. In the first case these molecules are divided in two families: the CD28/B7 family and the tumor necrosis factor (TNF) receptor family; in the second case they are divided into positive and negative costimulators belonging to the CD28/B7 family. The CD28/CD80-CD86 axis was the first positive costimulatory pathway to be identified. $CD4^+$ T cells constitutively express CD28 and its binding to CD80 (B7-1) and CD86 (B7-2) on APC favours T cells activation, cytokine secretion and survival (Carreno and Collins, 2002). Another member of the CD28/B7 family, but with negative costimulatory functions, is the cytotoxic T-lymphocyte antigen 4 (CTLA-4 or CD152). CTLA-4 is expressed by activated T cells and binds to CD80-CD86 with higher affinity than CD28. Upon CD80-CD86 engagement by CTLA-4, on one-hand APC down-modulate costimulatory molecules on their surface, on the other hand T cell functions and proliferative potential are blocked (Carreno and Collins, 2002). Programmed death 1 (Pd1), another member of the CD28/B7 family expressed by activated T cells, binds to PD-L1 and PD-L2 on APC and, as CTLA-4, has inhibitory

consequences on both T cells and APC, reducing survival, proliferation and cytokine secretion (Keir et al., 2008).

The TNFR family encompasses a lot of molecules crucial for the modulation of T cell and APC functions. Among these CD40 is one of the most studied, as its interaction with the corresponding ligand, CD40L, is indispensable for the full T cell activation and APC maturation (Elgueta et al., 2009). CD40L, a type II transmembrane protein, is expressed by T cells within 1-2 hours upon activation. On the contrary the subset of memory cells up-regulate CD40L in a shorter time, in about 15 minutes, because these cells contain pre-formed CD40L and do not need its de novo synthesis (Elgueta et al., 2009). CD40 is type I transmembrane protein and is expressed by DC, B cells and monocytes (Elgueta et al., 2009). The CD40L/CD40 axis has bidirectional effects, promoting maturation of both T cells and APC. The main consequences of CD40 engagement in APC are: up-regulation of pro-survival genes like Bcl-XL, increased secretion of effector cytokines (IL-1, IL-12, IL-2, IL-6), up-regulation of costimulatory molecules (CD80 and CD86), more stable MHC/Ag complex. Moreover CD40-stimulated APC show more efficient induction of cytotoxic $CD8^+$ T cell response, improved ability to stimulate immunoglobulin production by plasma cells, GC formation and memory B cells differentiation (Casamayor-Palleja et al., 1995; van Kooten and Banchereau, 2000). On the other hand, upon CD40L/CD40 interaction, T cells receive stronger positive costimulatory signals, acquire fully effector functions and secrete high amount of cytokines (Casamayor-Palleja et al., 1995; van Kooten and Banchereau, 2000). The relevance of this pathway in regulating immune responses was highlighted by studies performed both in human and mouse on autoimmune diseases (Peters et al., 2009) and tumors (Fransen et al., 2011; Higham et al., 2010; Hussein et al., 2010).

1.3.3 Differentiation of memory T cells

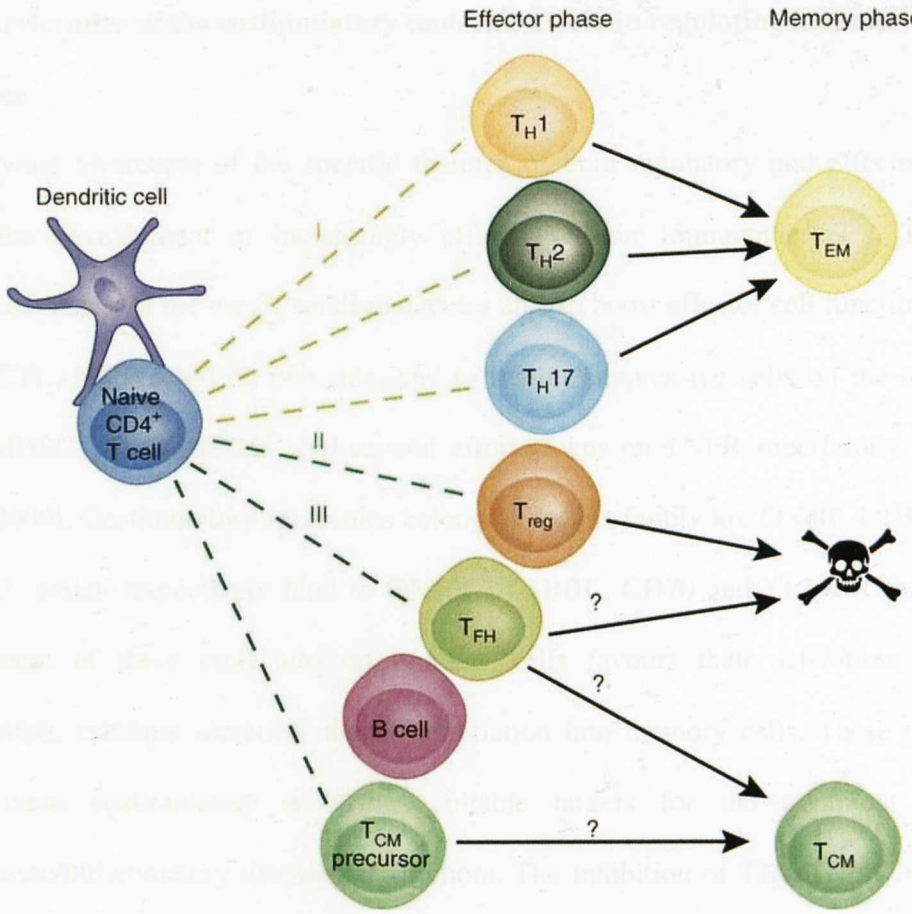
Upon activation, the majority of T cells die within 1-2 weeks, however some of these cells differentiate into long-lived memory T cells, which ensure strong and rapid immune responses in case of subsequent interactions with the same antigen. Memory T cells can be distinguished in central memory (Tcm) and effector memory (Tem) cells, according to anatomical localization, proliferation and cytokine secretion (Lanzavecchia and Sallusto, 2005; Sallusto et al., 1999). The existence of these two memory subsets was confirmed both in humans (Sallusto et al., 1999) and in mice (Reinhardt et al., 2001; Roman et al., 2002). Both Tem and Tcm are characterized by CD45RO and CD44 expression, markers of memory phenotype (Swain, 1994); Tcm also express the chemokine receptor CCR7 and L-selectin (CD62L), which let them localize into secondary lymphoid organs (Arbones et al., 1994; Willimann et al., 1998). Conversely, Tem are CCR7 and CD62L negative and mainly localize in non-lymphoid peripheral tissues, rapidly moving to inflamed sites (Sallusto et al., 1999). Both memory subsets display a more rapid activation upon CD3 stimulation and a higher expression of CD40L in comparison to Tn. During immune responses to previously encountered Ag, Tem respond more quickly than Tcm and produce higher amount of effector cytokines, like IL-4, IL-5 and IFNγ, but secrete less IL-2 (Sallusto et al., 1999). In a recent work it was also demonstrated that activated Tem enter lymph nodes, constitutively express CD40L and activate DC, resident into chronic reactive lymph nodes, in an antigen independent manner (Martin-Fontecha et al., 2008). In summary, during second antigen challenge, Tcm remain distributed in peripheral lymphoid tissues, efficiently proliferate, maintain protection in the long term, secrete considerable amount of IL-2 and low quantities of effector cytokines. On the contrary, Tem rapidly move toward the tissue that is the site of inflammation, immediately secrete abundant effector cytokines, but their proliferative capacity is limited (Pepper and Jenkins, 2011;

Sallusto et al., 2004; Sallusto et al., 1999). In the mouse, memory cells need IL-7 and TCR signals to survive (Seddon et al., 2003), while in humans $CD4^+$ memory T cells survive in response to IL-7 and IL-15 (Geginat et al., 2001).

Very interesting questions are: i) how $CD4^+$ memory T cells are generated after the peak of an immune response, ii) which is the relationship between Tcm and Tem and iii) which are the signals that drive their differentiation? Upon activation, Tn differentiate into one of the Th subsets, depending on the stimuli they receive and the cytokine milieu in which they are located (Zhu and Paul, 2010). Many pieces of evidence show that, when antigen stimulation during the first immune response is strong enough to commit Tn toward Th1 or Th2 lineage, Tem differentiate from both Th1 and Th2 cells. On the contrary Tcm develop from activated but not committed precursors (Pepper and Jenkins, 2011). In regard to Th17 and Treg the question is not yet well understood. It seems that Th17 give rise to short-term living memory cells, probably because Th17 cells, during the later phases of inflammation, can differentiate into Th1 cells (Pepper and Jenkins, 2011). Recently it was shown that, in response to autoimmune attack, Treg develop a kind of "regulatory tissue memory", such to resolve quickly and better subsequent immune responses against self-antigens (Rosenblum et al., 2011). However it is not clear whether Treg differentiate or not into memory cells, since several pieces of evidence show that Treg lose Foxp3 expression and acquire a Th1 or a Th2 memory phenotype upon activation (Hansmann et al., 2011; Zhou et al., 2009c). Tfh develop during immune responses, form GC and survive until GC are functional, thus hardly Tfh differentiate into memory cells. However very recent data in humans show that circulating $CD4^+CXCR5^+$ T cells may represent the central memory compartment of Tfh because of their ability to stimulate B cells to produce antibodies in an IL-21 dependent manner (Morita et al., 2011). The development of Tcm from Tfh may be dependent on signals provided by B cells via ICOS-ICOSL interaction (Rasheed et al.,

2006), which seems to be involved also in Tcm differentiation from uncommitted Th precursors (Pepper and Jenkins, 2011).

The more recent model proposed to explain the generation of Tcm and Tem generation is based on the different strength and duration of TCR/MHC-Ag interaction. Upon strong TCR signaling, Tn differentiate into a specific Th subset, and those cells, which survive to the peak of inflammation, become Tem cells. Tn that receive a lower TCR stimulation and interact with B cells without acquiring a specific Th commitment develop into Tcm (Pepper and Jenkins, 2011).



(From Pepper M., 2011)

Figure 1.5: CD4⁺ memory T cell development. Generation of Tem and Tcm from CD4⁺ Tn depend on the strength and duration of TCR stimulation via MHC/Ag complex on APC. When Tn differentiate into a specific Th subset (I) upon strong TCR stimulation, they give rise to Tem. Treg, which lose Foxp3 expression upon activation, die (II) or convert into Th1/Th2 cells. Weakly activated T cells, which do not acquire a well defined Th phenotype, and Tfh (III), upon interaction with B cells, become Tcm lymphocytes.

1.4 The relevance of the costimulatory molecule OX40 in regulating immune responses

The growing awareness of the specific features of both regulatory and effector T cells allows the development of increasingly efficient cancer immunotherapies. The ideal treatment consists in the use of small molecules able to boost effector cell functions ($CD4^+$ T cell, CTL, NKT cells) on one side, and to inhibit suppressive cells on the other side (Treg, MDSC). To reach this goal several efforts focus on TNFR superfamily members (Croft, 2009). Costimulatory molecules belonging to this family are OX40, 4-1BB, CD27 and DR3, which respectively bind to OX40L, 4-1BBL, CD70 and TL1A (Croft, 2009). Engagement of these molecules on immune cells favours their activation, survival, proliferation, cytokine secretion and differentiation into memory cells. These properties render these costimulatory molecules suitable targets for the treatment of both autoimmune/inflammatory diseases and tumors. The inhibition of TNF/TNFR interaction dampens immune responses with beneficial consequences for the host in case of excessive activation of the immune system. On the contrary, the improvement of these pathways allows the mounting of anti-tumor responses bypassing the suppression exerted by immune-regulatory and tumor cells (Croft, 2009).

In this regard OX40 (CD134) instigates particular interest. $CD4^+$ and $CD8^+$ T cells up-regulate it only upon activation and the interaction with OX40L on APC provides activator stimuli (Croft et al., 2009). On the contrary Treg constitutively express OX40, but its engagement has detrimental consequences on Treg biology, "contra-suppressing" their inhibitory functions (Valzasina et al., 2005). In this view it is reasonable to consider OX40 a key element for the development of a successful cancer immunotherapy, as a single molecule is concurrently able to boost cells endowed with anti-tumoral functions, and to block Treg, which promote tumor growth

1.4.1 OX40 and OX40L expression

At the end of the eighties Williams and his group were the first to identify a mAb recognizing OX40 (Paterson et al., 1987), and they also observed that stimulation via OX40 promotes T cell proliferation. Few years later it was demonstrated that OX40 is a member of the tumor necrosis factor receptor (TNFR) superfamily (Mallett et al., 1990). During the same period the ligand of OX40 was also identified: OX40L (CD252) (Tanaka et al., 1985), member of the TNF superfamily.

OX40 is expressed by activated $CD4^+$ and $CD8^+$ T cells, NK and NKT cells, neutrophils, and it is constitutively expressed by Treg (Croft et al., 2009; Redmond et al., 2009). Although OX40 is found on several cells, the majority of the studies were focused on the effects of OX40 engagement on T cells. The up-regulation of OX40 on non-regulatory lymphocytes occurs in the subsequent 24 hours upon TCR stimulation, persists for the next 4-5 days, and is down modulated on memory cells. The expression level of OX40 is proportional to TCR signal: stronger is the stimulation via TCR, higher is the expression of OX40 (Croft et al., 2009; Redmond et al., 2009). Although the activation via TCR is indispensable for the initial OX40 expression, other signals modulate the level and the duration of OX40 up-regulation, for instance the costimulatory axis CD28/CD80-CD86 (Walker et al., 1999), and cytokines like TNF (Hamano et al., 2011), IL-2 (Rogers et al., 2001), IL-4 (Toennies et al., 2004) and IL-1 (Nakae et al., 2001). The stimulatory signals provided by OX40 are not required to initiate the immune response, but are relevant to sustain and prolong the activation status of T cells (Song et al., 2005; Vasilevsky et al., 2011).

B cells, DC, NK cells, $CD4^+CD3^-$ accessory cells and macrophages express OX40L during immune responses, but not in the resting state (Croft et al., 2009; Redmond et al., 2009). CD40 engagement, LPS stimulation, TSLP and IL-18 are crucial signals in regulating the

extent of OX40L expression (Murata et al., 2000; Ohshima et al., 1997). The kinetics of OX40L is similar to that of OX40, as it is induced within 24 hours after APC activation. It has been recently demonstrated that also mast cells (MC) express OX40L and are able to tune T cell responses in an OX40/OX40L fashion manner (Nakae et al., 2006; Piconese et al., 2009). Also T cells could express OX40L in the later phases of activation, further boosting the activation status of inflammatory cells (Mendel and Shevach, 2006).

1.4.2 Modulation of Treg biology by OX40

Gene expression comparison between $CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ Teff cells provided the first evidence that OX40 is constitutively expressed by Treg (Gavin et al., 2002; McHugh and Shevach, 2002). Subsequent studies confirmed these data and also demonstrated that upon TCR engagement OX40 expression level is further increased on Treg (Valzasina et al., 2005). Functional experiments point out three different aspects of OX40 in Treg biology: i) suppressive functions, ii) homeostasis and survival, iii) iTreg differentiation.

1.4.2.1 OX40 inhibits Treg suppressive functions

The inhibitory effect of OX40 engagement on Treg functions was evaluated in both *in vitro* and *in vivo* settings. The first issue under investigation is whether OX40 triggering abrogates Treg suppressive functions or the addition of an OX40 agonist mAb (OX86) to a co-culture of Treg and Teff renders Teff resistant to the control exerted by Treg. In 2004 Takeda et al. showed that in a co-culture system of wt Treg and $OX40^{-/-}$ Teff, in the presence of OX86 Treg efficiently suppressed Teff proliferation, indicating that the main target of OX86 were Teff and not Treg (Takeda et al., 2004). On the contrary, the following year, Valzasina et al. (Valzasina et al., 2005) demonstrated that OX86 directly

hampers Treg abilities by co-culturing rat $CD4^+$ T cells (which do not bind OX86) and mouse Treg. Another piece of evidence that OX40 triggering is crucial in regulating the suppression exerted by Treg was provided by Vu et al. in 2007 (Vu et al., 2007), using OX40Ltg APC to stimulate sorted $CD4^+GFP(Foxp3)^+$ or $CD4^+OX40^{-/-}GFP(Foxp3)^+$. These experiments demonstrated that OX40 engagement impairs the suppressive functions of wt, but not OX40-deficient, Treg against both OX40-sufficient and -deficient responder T cells (Vu et al., 2007).

These observations were confirmed also in different mouse disease models. In a model of graft versus host disease (GVHD), in which the pathogenic effect of $CD4^+CD25^-$ Teff was abrogated by the co-injection of wt Treg, the *in vitro* pre-incubation of Treg with OX86 was shown to completely abolish the protective effect of Treg (Valzasina et al., 2005). Same results were obtained in experiments of skin transplantation (Vu et al., 2007). In this model $Rag^{-/-}$ mice were reconstituted with sorted $CD4^+OX40^{-/-}GFP(Foxp3)^-$ Teff cells alone or co-injected with sorted $CD4^+GFP(Foxp3)^+$. Thereafter mice were grafted with the full-thickness tail skin of fully MHC-mismatched mice. Some of the transplanted mice were treated with OX86 four times from the day of the graft. $Rag^{-/-}$ mice injected with Treg did not reject the skin allograft, but the treatment with OX86 significantly impaired the suppressive function of Treg and all the skin allografts were rejected (Vu et al., 2007).

1.4.2.2 OX40 in Treg homeostasis

Despite the negative signals provided by OX40 triggering to Treg suppression capabilities, it has been recently demonstrated that OX40 is also implicated in Treg homeostasis and survival. Studies done in $OX40^{-/-}$ or $OX40L^{-/-}$ mice displayed that the OX40/OX40L interaction is dispensable for Treg development, as $CD4^+Foxp3^+$ cells are still present in these mice (even if young mice, but not old mice, have less Treg in both thymus and

spleen, compared to aged-matched wt mice) (Takeda et al., 2004). Although $OX40^{-/-}$ Treg do not display lower suppressive functions compared to OX40 sufficient Treg (Vu et al., 2007), their proliferative potential in lymphopenic condition is impaired (Takeda et al., 2004). On the contrary Treg transferred into OX40L transgenic mice efficiently expand (Takeda et al., 2004). Very recent data demonstrate that OX40 expression is necessary for both Treg competitive fitness in lymphopenic hosts and for Treg survival and proliferation in an IL-2 dependent manner (Piconese et al., 2010; Xiao et al., 2012). Xiao and colleagues showed that OX40 triggering in physiological conditions promotes the differentiation and expansion of Treg, however these regulatory cells have an exhausted phenotype and display weak inhibitory functions. This condition could be reverted by the double stimulation via OX40 and the addition of exogenous IL-2. In fact, OX40 engagement alone promotes Stat5 phosphorylation and sensitivity to IL-2, generating paucity of available IL-2, which is necessary for Treg survival. Exogenous administration of IL-2, in concomitance to OX40 triggering, assure for the development of fully differentiated and functional Treg (Xiao et al., 2012).

$CD4^+Foxp3^+$ cells have a crucial role in keeping under control immune responses in mucosal tissues, like gut and lung. In physiological conditions, OX40 expression is required for the accumulation of Treg in the gut, and during inflammatory responses provides survival signals and prevents Treg activation-induced cell death. In such a way, assuring Treg persistence, OX40 avoids excessive immune activation (Griseri et al., 2010).

1.4.2.3 OX40 Triggering blocks iTreg differentiation

A different issue deals with the involvement of OX40 triggering in tuning the differentiation of iTreg. As previously described, during immune responses, $CD4^+$ non-regulatory cells in the presence of high concentration of TGFβ and low TCR stimulation

could acquire Foxp3 expression and differentiate into iTreg, indistinguishable from nTreg (Wing et al., 2006). This conversion is reinforced also by IL-2 and retinoic acid (Mucida et al., 2009). Other subsets of iTreg include IL-10-secreting Tr1 regulatory cells and TGFβ-producing Th3 cells (Chen et al., 1994). Many studies demonstrated that OX40 triggering on Teff down-regulates Foxp3 expression and dampens iTreg differentiation (So and Croft, 2007; Xiao et al., 2008). *In vitro* experiments of conversion of wt or $OX40^{-/-}$ $CD4^+CD25^-$ Teff cells into iTreg showed that OX40 antagonizes Foxp3 expression by blocking TGFβ signals (So and Croft, 2007). This property of OX40 was confirmed also in an *in vivo* model of airway tolerance. In this model the intranasal administration of the Ag allows the development of tolerance and the differentiation of iTreg, with low concentration of IL-4 and IFNγ. The co-administration of LPS with the Ag prevents the tolerization and leads to the development of the disease. Upon LPS stimulation, DC and B cells express OX40L, which, interacting with OX40 on T cells, impedes their differentiation into iTreg and causes the accumulation of inflammatory cytokines in the lung microenvironment (Duan et al., 2008). Another mechanism by which OX40 prevents the development of iTreg was described by Xiao et colleagues (Xiao et al., 2008). In their study, OX40L tg mice-derived T cells oppose stronger resistance to Foxp3 expression upon TGFβ stimulation compared to non regulatory T cells isolated from wt or $OX40^{-/-}$ mice. Analysis of T cell subsets revealed that in OX40L tg mice the fraction of $CD4^+CD44^{high}CD62L^{low}$ effector memory cells is significantly increased compared to wt mice. Intriguingly this memory population blocks the conversion of naïve T cells in iTreg in periphery by secreting large amount of IFNγ.

In the tumor microenvironment the conversion of non-regulatory $CD4^+$ T cells into Treg is a frequent event. In this immune-depressed microenvironment OX40 stimulation prevents the generation of iTreg, as demonstrated by Piconese et al. (Colombo and Piconese, 2007;

Piconese et al., 2008), thus representing an interesting candidate for the development of efficient cancer immunotherapy.

In addition OX40 ligation was also reported to impair both the de-novo differentiation of Tr1 cells from naïve and memory cells and the secretion of IL-10 by fully differentiated Tr1 cells (Ito et al., 2006).

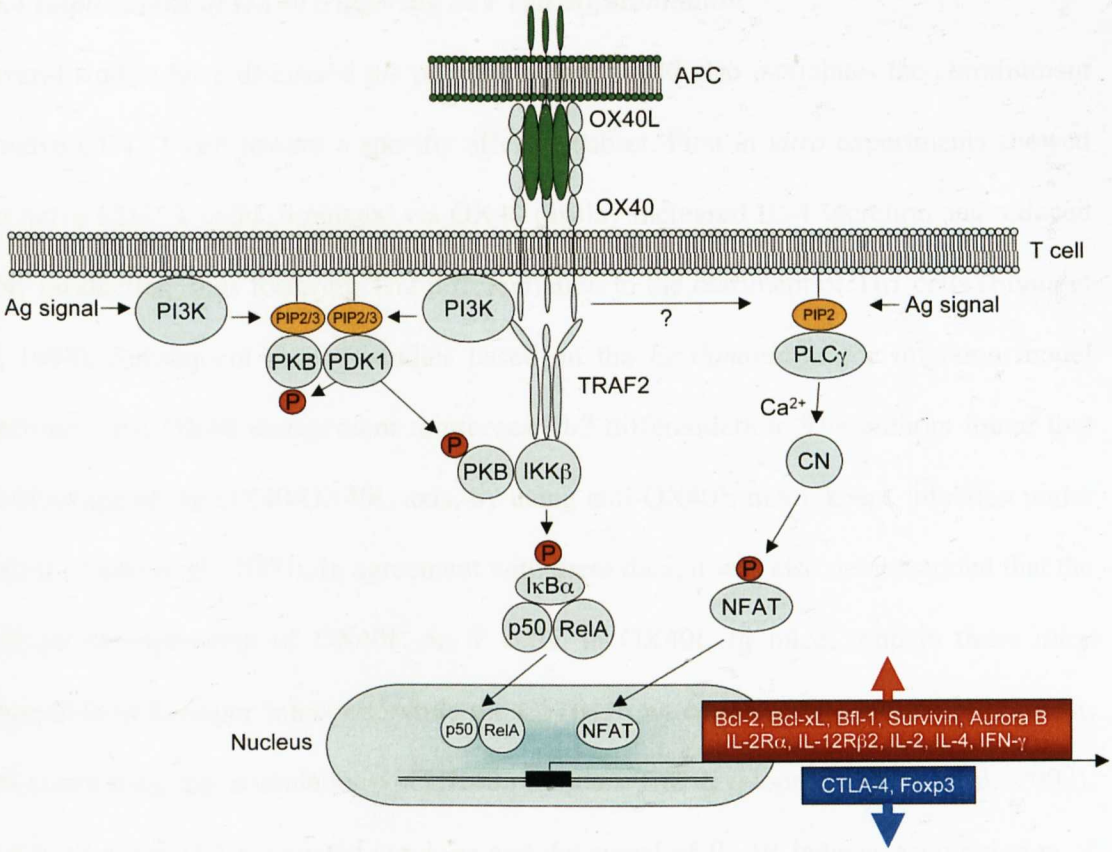
A more complex analysis about the roles of OX40 in the process of iTreg induction asserts that the commitment of T cells toward a specific subset in response to OX40 engagement depends on the local cytokine milieu (Ruby et al., 2009). This observation comes from the context of the experimental autoimmune encephalomyelitis (EAE) model. OX40 triggering during the priming phase of the disease favors the differentiation of Treg and prevents the onset of the symptoms. On the contrary the administration of the OX40 agonist in the later phase of EAE induces the development of inflammatory cells and the exacerbation of the disease.

1.4.3 Modulation of $CD4^+$ T cell functions by OX40

Unlike Treg, $CD4^+Foxp3^-$ cells express OX40 only upon TCR engagement and the expression level of OX40 is proportional to the strength of TCR signaling. Up-regulation of OX40 occurs within the first 24 hours following activation and persists for the next 4-5 days, also according to the presence in the microenvironment of other factors, like TNF (Hamano et al., 2011), IL-2 (Rogers et al., 2001), IL-4 (Toennies et al., 2004) and IL-1 (Nakae et al., 2001), which prolong OX40 expression. During immune responses OX40 plays a critical role in promoting clonal expansion of $CD4^+$ T cells, but it is not required during the initial phase of activation and proliferation. Upon activation $OX40^{-/-}$ T cells display a normal proliferation rate and differentiate into memory cells, however in the later phases of the immune response (day 12-13) their survival is critically compromised

(Gramaglia et al., 2000; Song et al., 2005). OX40 stimulation is required for the expression of genes that regulate cell proliferation and survival, like survivin (Song et al., 2005), Bcl-xL and Bcl-2 (Colombo and Piconese, 2007; Piconese et al., 2008; Rogers et al., 2001; Song et al., 2008). OX40 stimulation prevents and reverts T cell anergic state (Bansal-Pakala et al., 2001).

The crystal structure of the human OX40/OX40L complex has been described by Campaan and colleagues (Compaan and Hymowitz, 2006; Song et al., 2005). OX40 organizes in a trimeric complex, which binds to three copies of OX40L, forming a quaternary hexamer complex. OX40 engagement induces the activation of both the canonical NF-KB1 (Song et al., 2008) and the PI3K/PKB (So et al., 2011a) pathways. Following the interaction with OX40L, the intracellular domain of OX40 binds to TNFR-associated factor (TRAF) 2 and TRAF5, which are indispensable mediators for the induction of the survival signals provided by OX40 via NF-kB1 activation (Kawamata et al., 1998; Prell et al., 2003).



(from Croft M. et al. 2009)

Figure 1.6: OX40/OX40L pathway. The intracellular domain of OX40 binds to TNFR-associated factor (TRAF) 2, which forms a complex including IKKα, IKKβ, PI3K and PKB (Akt) and activates the nuclear factor kB1 (NF-kB1). OX40 also promotes the intracellular up-take of calcium upon TCR stimulation, favouring the nuclear localization of nuclear factor of activated T cells (NFAT). These two pathways (NF-KB1 and NFAT) enhance the transcription of genes involved in regulating cell proliferation, survival, cytokine secretion and cytokine receptor expression.

1.4.4 Implications of OX40 triggering in T cell differentiation

Several studies have discussed the possibility that OX40 also modulates the commitment of naïve $CD4^+$ T cell toward a specific effector subset. First *in vitro* experiments showed that naïve $CD4^+$ T cells stimulated via OX40 display increased IL-4 secretion and reduced IFNγ production, thus fostering Th2 differentiation to the detriment of Th1 cells (Flynn et al., 1998). Subsequent *in vivo* studies based on the *Leishmania major* infection model confirmed that OX40 engagement reinforces Th2 differentiation. The authors found that the blockage of the OX40/OX40L axis, by using anti-OX40L mAb, keeps infection under control (Akiba et al., 2000). In agreement with these data, it was also demonstrated that the constitutive expression of OX40L on T cells, in OX40L tg mice, renders these mice susceptible to *L. major* infection, while the C57BL/6 wt counterpart is normally resistant, thus confirming that stimulation via OX40 promotes Th2 development (Ishii et al., 2003). IL-4 is a typical Th2-associated cytokine and the signal of IL-4R induces accumulation of the transcriptor factor GATA-3 in the nucleus of T cells, which acquire a Th2 phenotype. So and colleagues showed that, in the absence of an external source of IL-4, OX40 provides the necessary stimuli to promote GATA-3 nuclear localization, which in turn guides T cells along the Th2 differentiation process (So et al., 2006). In a study conducted on human DC, it was demonstrated that TSLP-stimulated DC up-regulate OX40L but do not secrete IL-12. Such DC promote the differentiation of Th2 cells able to secrete TNFα, IL-4, IL-5 and IL-13, but not IL-10. However the addition of exogenous IL-12 shifts T cell differentiation toward a Th1 phenotype (Ito et al., 2005). Other studies have highlighted that in different contexts OX40 could support the development of other Th subsets beside Th2 (De Smedt et al., 2002; Rogers and Croft, 2000). Recently it has been also described that mast cells (MC) constitutively express OX40L and are able to drive T differentiation toward a Th17 phenotype (Piconese et al., 2009). In an *in vitro* system in which MC, Treg

and Teff are co-cultured, Treg are able to suppress MC degranulation in an OX40/OX40L dependent manner. Otherwise MC, through OX40 ligation, inhibit Treg suppressive functions, and render Teff more resistant to Treg suppression. In this study the more relevant aspect is that Treg, in presence of MC, lose their ability to suppress Teff, but continue to block the secretion of Th1 (IFNγ) and Th2 (IL-4) cytokines. The reduced suppression exerted by Treg is mediated by IL-6, released from both MC and Teff, and IL-6 itself sustains OX40L expression on MC. In this environment, rich in IL-6 and poor in IFNγ and IL-4, MC, via OX40L, induce both Treg and Teff to acquire an inflammatory Th17 phenotype.

This evidence suggests that the OX40/OX40L does not drive T cell differentiation in only one specific direction, but amplifies immune responses according to the particular cytokine milieu.

1.4.5 Differentiation of memory T cells is influenced by OX40

Naïve T cells, upon Ag recognition, activate and differentiate into specific subsets of Th cells. A small proportion of these lymphocytes give rise to the pool of memory cells, which assure rapid and protective immune response during subsequent encountering with the same Ag. As previously described, memory T cells can be classified in two groups according to CD44 and CD62L expression. Tem, which are $CD4^{+}CD44^{high}CD62L^{low}$, are mainly localized in non-lymphoid tissues, while $CD4^{+}CD44^{high}CD62L^{high}$ Tcm preferentially accumulate in secondary lymphoid organs (Lanzavecchia and Sallusto, 2005; Sallusto et al., 1999). The signals that guide activated Teff to differentiate into Tem or Tcm cells rely on the strength and the length of TCR/MHC-Ag interaction. The commitment toward a Tcm phenotype is induced upon weak and short TCR/MHC-Ag

signals. Conversely, the differentiation of Tem cells requires strong and long-lasting interaction between APC and T cells (Pepper and Jenkins, 2011).

The OX40/OX40L axis is relevant for the development of Tem, while being dispensable for Tcm development. This discrepancy concerning the costimulation via OX40/OX40L is in agreement with the time course of T cells-APC interaction for the specification of a Tcm or a Tem phenotype. OX40L on APC is expressed only in the later phase of the immune response (Croft et al., 2009; Redmond et al., 2009), thus Tcm, which shortly interact with APC, do not receive signals via OX40. On the contrary Tem, which keep prolonged interaction with APC for their fully differentiation, are in the optimal condition to receive positive stimulation in a OX40/OX40L-dependent fashion.

First experiments performed in OX40L tg mice, which constitutively express OX40L on T cells, showed that the OX40/OX40L interaction significantly increases the number of splenic Tem compared to age-matched wt mice. Tem generated in OX40L tg mice are efficiently activated in response to Ag also in absence of adjuvant, and the OX40 axis protects Tem from activation-induced cell death (AICD) (Murata et al., 2002). Complementary results were obtained in OX40 deficient mice (Soroosh et al., 2007). Soroosh and colleagues found low numbers of Tem in lymph nodes, spleen, lung, liver, lamina propria of the colon and in peritoneal cavity of OX40 deficient mice compared to age-matched wt mice. On the contrary no difference was observed between the two mouse strains for Tcm population. To better evaluate the relevance of OX40 in Tem/Tcm differentiation they performed experiments of adoptive transfer using OTII cells isolated from OX40 deficient or sufficient mice. OX40 signaling was indispensable for the long-lasting survival of Tem, while being dispensable for Tcm differentiation and maintenance over time (Soroosh et al., 2007). The OX40/OX40L axis promotes Tem generation also inhibiting the development of Treg (Xiao et al., 2008). Indeed Tem isolated from OX40L

tg mice do not convert into Foxp3$^+$ regulatory T cells, although cultured in the presence of adequate amounts of TGFβ. Tcm developed in OX40 tg mice are also able to block the conversion of naïve T cells into Treg by secreting high amount of IFNγ (Xiao et al., 2008). Collectively these data suggest that OX40 is not involved in the early phase of T cells commitment toward Tcm/Tem phenotype, but is crucial in the subsequent step, providing survival and proliferative signals to early differentiated Tem, thus warranting their long-last survival.

OX40 is transiently expressed on effector/memory T cells, and is rapidly down modulated at the end of the immune response. However the kinetics of OX40 expression on memory cells is faster than on recently activated T cells (Gramaglia et al., 1998) and signals provided via this pathway are necessary for memory T cells reactivation and acquisition of effector functions. In a model of memory Th2-induced asthma (Salek-Ardakani et al., 2003), OX40/OX40L axis was demonstrated to be required not only for the generation of memory Th2 cells, but also for their reactivation upon second Ag exposure. Indeed, blocking the OX40/OX40L interaction during Ag rechallenge prevents the accumulation of inflammatory cells in lung tissue. Moreover, Ag-specific OX40-deficient Th2 cells fail to induce inflammation because they do not receive appropriate survival stimuli (Salek-Ardakani et al., 2003). Similar results were obtained in a model of skin allograft rejection, where the blockage of CD28, CD154 and OX40 pathways significantly prolonged skin allograft acceptance (Vu et al., 2006).

Considering the several consequences of OX40/OX40L axis in regulating Treg and Teff biology it is reasonable to consider these molecules interesting targets for the development of therapies for autoimmune/inflammatory diseases and tumors, blocking or reinforcing its signal according to the desired outcome.

1.4.6 OX40/OX40L in autoimmune diseases and inflammation

Autoimmune diseases are caused by an excessive and uncontrolled activation of the immune system against self-antigens. The ideal therapy for these pathologies aims to block only the cells responsible for the disease onset without modifying the functions of the non-pathogenic immune cells. To this aim, OX40 appears to be the adequate candidate, since only activated, and presumably pathogenic, T cells express it. Thus the blockage of the OX40/OX40L axis would suppress only these cells, without inducing a general immunosuppressive environment. The first evidence that OX40 is implicated in the onset of autoimmune diseases was provided by Weinberg and collaborators in 1996 (Weinberg et al., 1996a; Weinberg et al., 1996b) in EAE. They showed that auto-reactive T cells express OX40 and their depletion, using an OX40-immunotoxin, ameliorates the course of the pathology. The same study also showed that OX40 is expressed by auto-reactive T cells in the peripheral blood of patients affected by GVHD or rheumatoid arthritis (RA). Few years later the same group demonstrated that blocking *in vivo* the OX40/OX40L axis using an OX-40R:Ig-Fc chimeric protein reduces the severity of EAE (Weinberg et al., 1999). The relevance of this pathway in EAE outcome was also proved by using OX40L-deficient or OX40L tg mice (Ndhlovu et al., 2001). In absence of OX40L mice develop milder EAE compared to wt mice, while in OX40L tg mice the course of the disease is more severe. Mirroring the use of the OX-40R:Ig-Fc chimeric protein, the administration of a mAb antagonistic for OX40L (MR134L) reduces the severity of EAE, actively or passively (by adoptive transfer) induced (Nohara et al., 2001). The same mAb (MR134L) was found efficient also in the cure of acute GVHD (Tsukada et al., 2000). On the contrary, the use of an agonistic anti-OX40 Ab (M5) accelerates GVHD progression and lethality (Blazar et al., 2003). These results could be extended to other disease models, like skin allograft, where the MR134L mAb, in combination with anti-CD154 (MR1) and anti-

CTLA4Ig, prolongs skin graft acceptance (Demirci et al., 2004). In patients affected by RA, OX40 is expressed by T cells in the synovial fluid, while OX40L is up-regulated by cell localized in the synovial tissue. Treatment of mice affected by collagen-induced arthritis (CIA) with anti-OX40L (RM134L) mAb ameliorates disease severity (Yoshioka et al., 2000). In a mouse model of antigen-induced uveitis, treatment with a blocking anti-OX4L Ab (18269) or OX86 ameliorates or worsens the severity of ocular inflammation, respectively. In particular it was demonstrated that OX40 signals enhances the secretion of the inflammatory Th17-derived cytokines and also contributes to the stability of this T cell subsets by promoting IL-21 production (Zhang et al., 2010). The OX40/OX40L axis is also implicated in the development of autoimmune type-1 diabetes (T1D) (Bresson et al., 2011; Martin-Orozco et al., 2003; Pakala et al., 2004). Interestingly, a very recent study has showed that the administration of OX86 to non-obese diabetic (NOD) mice reduces the incidence of T1D, enhancing the development of both $CD4^+Foxp3^+$ Treg and $CD4^+Foxp3^-LAP^+$ suppressive cells (LAP: latency associated peptide) (Bresson et al., 2011). In colitis, the administration of OX40-Ig fusion protein or OX40L-Ig fusion protein has opposite consequences on the progression of the disease, blocking or promoting inflammation, respectively. These findings were also confirmed using anti-CD134 mAb and OX40L tg or OX40 deficient T cells (Higgins et al., 1999; Malmstrom et al., 2001). Moreover, OX40 expression on Treg was demonstrated to be necessary for the resolution of colitis (Griseri et al., 2010; Piconese et al., 2010). Indeed OX40-deficient Treg inefficiently accumulate in the colon, are more susceptible to AICD (Griseri et al., 2010), and their impaired proliferation renders them unable to control Teff activation (Piconese et al., 2010). The crucial role exerted by OX40 in the onset of autoimmune disordes was recently demonstrated using the scurfy mouse model. These mice lack Foxp3 and spontaneously develop severe autoimmune pathologies within the first weeks of life. However scurfy

mice backcrossed with $OX40^{-/-}$ mice show a significantly delay in the onset of autoimmune syndromes (Gaspal et al., 2011). The phenotype further ameliorates in mice double KO for both OX40 and CD30 (Gaspal et al., 2011).

1.4.7 OX40 triggering in cancer immunotherapies

Conversely to inflammatory/autoimmune diseases, in the immunosuppressive tumor microenvironment immune cells need to be boosted such to eliminate transformed cells. OX40 may represent the right molecule to achieve this goal, thanks to its ability to activate effector T cells and inhibit Treg (Croft et al., 2009; Sugamura et al., 2004). Few years after the first evidence of OX40 expression on $CD4^+$ T cells in tumor and dLN (Vetto et al., 1997), OX40 was proposed as a prognostic marker in tumor (Sarff et al., 2008). The beneficial effect of OX40 stimulation in tumor mass was demonstrated by Weinberg and collaborators in 2000 (Weinberg et al., 2000). Mice bearing established tumors were systemically treated with OX-40L:Ig fusion protein or anti-OX40 (OX86). These treatments resulted in the eradication of the majority of tumors and increased the percentage of tumor-free mice. The efficacy of OX-40L:Ig fusion protein and OX86 was confirmed in four different mouse tumor models (B16/F10, MCA303, SM1 and CT26), perhaps the more immunogenic being the most responsive to the therapy (Weinberg et al., 2000). In this regard another study has better evaluated the environmental elements that impact on the efficacy of OX86 treatment (Kjaergaard et al., 2000). Indeed, the responsiveness to the therapy depends not only on tumor cell immunogenicity but also on the anatomical localization of tumor nodules, the tumor burden and the amount of OX40-expressing tumor-infiltrating $CD4^+$ and $CD8^+$ T cells (Kjaergaard et al., 2000). The combination of adoptive immunotherapy with the administration of OX86 and/or IL-2 showed that anti-OX40 mAb enhances the efficacy of the therapy regardless the

anatomical localization of the tumor, while IL-2 anti-tumoral function depends on the anatomical district of tumor location (Kjaergaard et al., 2001). OX40-based therapy mainly targets tumor-specific T cells and enhances the generation of tumor-antigen specific $CD4^+$ memory cells (Weinberg et al., 2000). It has been recently described that in tumor microenvironment also DC could express OX40 (Pardee et al., 2010). In MCA205 tumor-bearing mice, inoculated i.p. with OX40L-Fc, $CD80^+CD86^+CCR7^+$ DC migrate from the tumor nodule to the draining lymph node in a $CD4^+$T cells-independent manner. In the dLN T cells receive two activating signals: on one side OX40L-Fc induces the up-regulation of CXCR3, on the other side migrated DC promote T cell full activation. In such a context, competent $CXCR3^+$ T cell move toward the tumor microenvironment. Migration of T cells toward the tumor is facilitated by a reorganized vascular system; indeed, upon OX40L-Fc treatment, vascular endothelial cells up-regulate CXCL9, the ligand for CXCR3, and VCAM-1, which mediates the adhesion of leukocytes to endothelium (Pardee et al., 2010). To potentiate the anti-tumoral effect of the OX40/OX40L axis, anti-OX40 mAb or OX40L-Fc could be provided in combination with other immune-stimulatory molecules. Colon carcinoma C26 cells transduced with OX40L (C26/OX40L) display a delayed growth rate *in vivo* compared to the parental C26 cells. C26 cells engineered to express both OX40L and GM-CSF (C26/GM/OX40L) are rejected by the majority of mice (85%) in a CD40-dependent mechanism (Gri et al., 2003). Interestingly, vaccination of mice, bearing C26 lung metastasis, with irradiated C26/GM/OX40L cells cures about 83% of animals (Gri et al., 2003). Synergism between OX40 and GM-CSF in anti-tumor immunity was observed also in transplantable colon (CT26) and mammary (4T1) carcinomas (Ali et al., 2004) and in a mouse model of spontaneous mammary carcinogenesis (MMTV-neu) (Murata et al., 2006). Vaccination with GM-CSF secreting tumor cells in combination with OX40 stimulation displays stronger results compared to

the sole vaccination with GM-CSF. In the presence of an OX40 agonist mAb, $CD8^+$ T cells efficiently expand, the pool of tumor-specific $CD8^+$ T survives for a longer period and importantly $CD8^+$ lymphocytes become independent from $CD4^+$ T cell help (Murata et al., 2006). The expression of OX40 also on $CD8^+$ T cells renders them direct targets of OX40-based therapy. Coupled stimulation of OX40 and 4-1BB in established mouse sarcoma leads to rejection of tumors in a $CD8^+$ T cell-dependent manner (Lee et al., 2004). Cooperation between $CD4^+$ and $CD8^+$ lymphocytes in tumor is favoured by OX40 engagement on $CD4^+$ T cells (Pan et al., 2002). Triple therapy of established tumors with intra-tumoral administration of adenovirus-IL-12 (Ad-IL-12), agonist mAb for 4-1BB and OX40 results in high survival rate of mice. OX40-stimulated $CD4^+$ lymphocytes, in the presence of IL-12, acquire a Th1 phenotype and enhance the generation of $CD4^+$-dependent CTL and long-living memory cytotoxic cells, which assure protection against rechallenge with parental tumor cells (Pan et al., 2002). OX40-stimulated $CD4^+$ T cells up-regulate IL-12Rbeta2 and signals delivered via IL-12 favour cell survival in a Stat4-dependent manner (Ruby et al., 2008). Croft's group demonstrated the existence of an OX40-mediated cooperation between $CD4^+$ and $CD8^+$ T cells using the OVA-expressing E.G7 tumor model (Song et al., 2007). Transfer of tumor-specific OT-I cells and OX86, in tumor challenged mice, increases the survival rate of the animals. The beneficial consequences of this treatment are abrogated when OX40-sufficient OT-I cells are transferred into OX40-deficient hosts. These data suggest that OX86 mainly acts on $CD4^+$ T cells, which in turn favour the proliferation of OT-I transferred cells and their ability to secrete both of IL-2 and IFNγ (Song et al., 2007). A direct effect of OX40 on $CD8^+$ T cells was observed by the group of Weinberg (Redmond et al., 2007). *In vivo* stimulation of OT-I cells with OVA and anti-OX40 mAb leads to up-regulation of granzyme B and CD25. The same experiment performed in $CD4^+$ T cells depleted mice results in a lower

expression of both granzyme B and CD25 by Ag-stimulated $CD8^+$ T cells. Similar results were obtained using OX40-deficient OT-I cells. These data suggest that for the optimal activation of $CD8^+$ T cells OX40 ligation is necessary on both $CD4^+$ and $CD8^+$ lymphocytes, but also indicate that OX40 directly enhances $CD8^+$ T cells activation in a $CD4^+$ T cell-independent manner (Redmond et al., 2007).

Another strategy to potentiate T cell activation is the delivery of OX40L through adenoviral vectors, directly injected in tumor mass (Andarini et al., 2004) or transfected in tumoral (Andarini et al., 2004) or APC cells (Dannull et al., 2005). The growth of B16 melanoma, Lewis lung carcinoma and C26 colon carcinoma injected with a recombinant adenovirus vector expressing OX40L (AdOX40L) is significantly reduced as a consequence of an increased specific anti-tumor cytotoxic activity along with differentiation of $CD4^+$ cell toward a Th1 phenotype (Andarini et al., 2004). Same results were obtained transfecting B16 cells with the AdOX40L (Andarini et al., 2004). Costimulatory signals provided by mature APC are necessary for the full activation of T cells, however in tumor microenvironment the high concentration of immunosuppressive molecules retains APC in an inactive/tolerogenic state. The use of APC in vitro engineered to constitutively express costimulatory molecules could provide great advantage for successful immunotherapy. Bone marrow-derived dendritic cell (BM-DC) transfected with OX40L mRNA impair the growth of melanoma tumors mainly promoting Ag-specific $CD4^+$ T cell activation (Dannull et al., 2005). Vaccination of B16-F10 tumors with AdOX40L-transduced DC efficiently blocks tumor growth thanks to the double engagement of OX40 on both T and NKT cells (Zaini et al., 2007).

As previously described, OX40 exerts another relevant function in T cell biology: indeed, it is able to antagonize Treg suppressive functions, directly acting on $CD4^+Foxp3^+$ cells and indirectly contrasting the conversion of $CD4^+Foxp3^-$ lymphocytes into iTreg (Piconese

et al., 2008). Since the discovery that Treg worsened immune response to tumors, several attempts have been done to counteract their activity. In this regard the first experiments have focused on depletion of Treg by targeting CD25 with the mAb PC61 (Onizuka et al., 1999; Shimizu et al., 1999). Although this treatment has shown efficacy in some mouse tumor models, it was ineffective for the cure of established tumors (Curtin et al., 2008). The criticism of this treatment is that in naïve mice, before tumor challenge, only Treg express CD25 and are suitable target for PC61. In the presence of established tumors, also Teff up-regulate CD25, so PC61 depletes not only Treg but also activated effector T cells (Betts et al., 2007; Onizuka et al., 1999). In addition, in the tumor microenvironment, Teff are converted into iTreg, replacing the pool of depleted Treg within few weeks after PC61 treatment (Valzasina et al., 2006). According to these results a better strategy seems to be the functional inhibition of Treg rather than their depletion (Colombo and Piconese, 2007). In this setting Treg are functionally inactive but are sensed by the homeostatic control mechanisms, thus preventing the conversion of new iTreg. OX40, constitutively expressed by Treg and even at higher level by tumor-associated Treg, may be the correct molecule to reach this scope. As previously described OX40 engagement suppresses Treg both *in vitro* and *in vivo* (Valzasina et al., 2005; Vu et al., 2007). Intra-tumoral administration of OX86 promotes tumor rejection in different mouse tumor models (colon carcinoma, fibrosarcoma and mammary carcinoma and adenocarcinoma) (Piconese et al., 2008), by reducing the control exerted by Treg on effector cells and favouring the migration of DC from tumor to the dLN. The efficacy of OX86 treatment requires OX40 engagement also on Teff, and the presence of $CD8^+$ T cells (Piconese et al., 2008). Combined therapy with cyclophosphamide (CTX) and OX86 reduces the growth of B16 melanoma tumors and increases the rate of surviving tumor-bearing mice at day 50 after tumor challenge (Hirschhorn-Cymerman et al., 2009). Upon CTX treatment Treg further up-regulate OX40,

thus becoming the preferential target of OX86. Injection of CTX+OX86 causes Treg specific hyper-activation and subsequent death. The lower amount of tumor-associated Treg favours the accumulation of $CD8^+$ T cells and increases the Teff/Treg ratio (Hirschhorn-Cymerman et al., 2009), generating a permissive environment for the development of anti-tumor response. Triple treatment of A20 lymphoma with CpG+anti-OX40+anti-CTLA4 impairs tumor growth by reducing tumor-infiltrating Treg and promoting the activation of $CD4^+$ and $CD8^+$ effector cells (Houot and Levy, 2009)

Table 1.1: OX40 in autoimmune and inflammatory diseases

Model	Mouse/treatment	Disease symptoms	Ref
EAE	OX40-immunotoxin	Inhibition	(Weinberg et al., 1996a)
	OX-40R:Ig-Fc	Inhibition	(Weinberg et al., 1999)
	Anti-OX40L (MR134L)	Inhibition	(Nohara et al., 2001)
	$OX40L^{-/-}$ or $OX40^{-/-}$ mice	Inhibition	(Ndhlovu et al., 2001)
	OX40L tg mice	Exacerbation	(Ndhlovu et al., 2001)
GVHD	Anti-OX40L (MR134L)	Inhibition	(Tsukada et al., 2000)
	$OX40^{-/-}$ mice	Inhibition	(Blazar et al., 2003)
	Anti-OX40 (M5)	Exacerbation	(Blazar et al., 2003)
Arthritis	Anti-OX40L (MR134L)	Inhibition	(Yoshioka et al., 2000)
	Toxin-conjugated anti-CD134 liposome	Inhibition	(Boot et al., 2005)
Colitis	OX40-IgG fusion protein	Inhibition	(Higgins et al., 1999)
	Anti-OX40L (MR134L)	Inhibition	(Malmstrom et al., 2001)
Diabetes	$OX40L^{-/-}$ mice	Inhibition	(Martin-Orozco et al., 2003)
	Antagonistic OX40L mAb	Inhibition	(Pakala et al., 2004)
Asthma and allergic inflammation	$OX40L^{-/-}$ or $OX40^{-/-}$ mice	Inhibition	(Jember et al., 2001)
	Anti-OX40L (MR134L)	Inhibition	(Arestides et al., 2002; Hoshino et al., 2003)
Uveitis	Anti-OX40L (18260)	Inhibition	(Zhang et al., 2010)
Graft	Anti-OX40L (MR134L)	Inhibition	(Demirci et al., 2004)
	Antagonistic OX40-Ig fusion protein	Inhibition	(Curry et al., 2004)

Table1.2: OX40 stimulation in tumor

Treatment	Tumor	Ref
OX-40L:Ig fusion protein	Melanoma, colon carcinoma, sarcoma, breast cancer	(Weinberg et al., 2000)
OX86	Brest adenocarcinoma, colon carcinoma, fibrosarcoma	(Piconese et al., 2008)
OX86+IL-2	Sarcoma	(Kjaergaard et al., 2001)
Tumor cells overexpressing OX40L+GM-CSF	Colon carcinoma	(Gri et al., 2003)
GM-CSF vaccination+OX86	Mammary tumor	(Murata et al., 2006)
OX40L:Fc+DISC-HSV/mGM-CSF	Colon carcinoma, mammary tumors	(Ali et al., 2004)
OX86+4-1BB	Sarcoma	(Lee et al., 2004)
Lentivirus-IL-12+4-1BB+OX86	Colon carcinoma	(Pan et al., 2002)
Adenovirus-OX40L	Melanoma, lung carcinoma, colon carcinoma	(Andarini et al., 2004)
DC-OX40LmRNA	Melanoma	(Dannull et al., 2005)
DC-Adenovirus-OX40L	Melanoma	(Zaini et al., 2007)
CTX+OX86	Melanoma	(Hirschhorn-Cymerman et al., 2009)

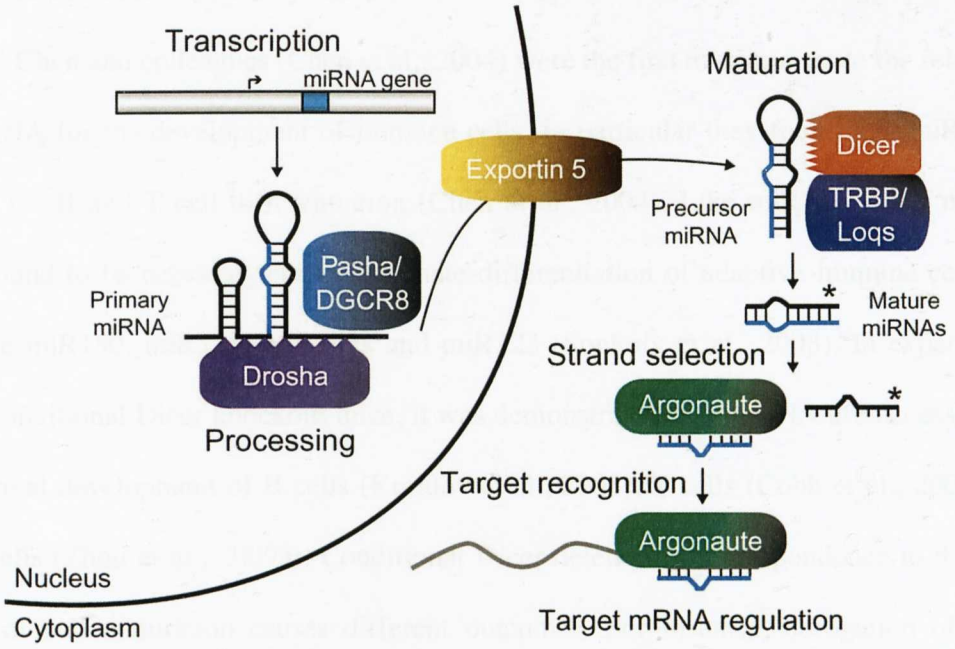
1.5 MicroRNA

MicroRNA (miRNA), described for the first time in 1993 in *C. elengans* by Ambros V. and his team (Lee et al., 1993), are endogenous 22 nt-long non-coding single strand (ss) RNA. miRNA play a critical role in the regulation of several biological processes, as they control the expression of most mRNA. Each miRNA has numerous mRNA as target, thus suggesting that a single miRNA may be involved in several different pathways, in addition a single mRNA is target of different miRNA, implying that miRNA may interact each other in order to control the gene expression (Garzon et al., 2010). Since their discovery miRNA have instigated great interest and always more researchers have focused their attention in order to understand how miRNA modulate cell differentiation, homeostasis and function. In this view it is not surprising that miRNA are involved in the onset of several pathologies, including dysfunctions of the immune system (Sonkoly et al., 2008), and tumors, as both tumor-suppressor genes and oncogenes are under the control of miRNA (Kong et al., 2012). For these reasons it is emerging the idea that specific miRNA expression profile can be used markers for different diseases (Kong et al., 2012).

1.5.1 miRNA biogenesis

miRNA derive from DNA sequence localized in both intragenic and intergenic regions. These sequences are transcribed into a primary transcript, named pri-miRNA, by RNA polymerase II (Pol II). The pri-miRNA is usually long 1-3 kb and is cleaved into a 60-70 nt stem loop intermediate (pre-miRNA) by the endonuclease Drosha, which is a RNAase III. The pre-miRNA is transported from the nucleus to the cytoplasm via exportin 5 and here Dicer, another RNAase III endonuclease, processes it into a double-strand (ds) RNA duplex, which contains the mature miRNA. This ds-RNA finally interacts with the RNA-induced silencing complex (RISC), where the Argonaute family proteins cleave the ds-

RNA duplex into the mature ss-miRNA. The complex ss-miRNA/RISC binds to the target mRNA by a partial sequence complementarity, between the nt 2-8 from the 5' UTR of the miRNA and the 3' UTR of the mRNA. When the complementarity between the miRNA and the mRNA is perfect, the mRNA is cleaved; on the contrary when the complementarity is partial there is translational repression (Kai and Pasquinelli, 2010). In addition to this canonical interaction, miRNA may also bind to the 5' UTR and to the ORF of target mRNA. These non-conventional interactions may promote gene expression, rather than block it (Garzon et al., 2010). Recently were described also miRNA able to directly bind to the DNA sequence into the nucleus, regulating gene expression at the transcriptional level (Khraiwesh et al., 2010).

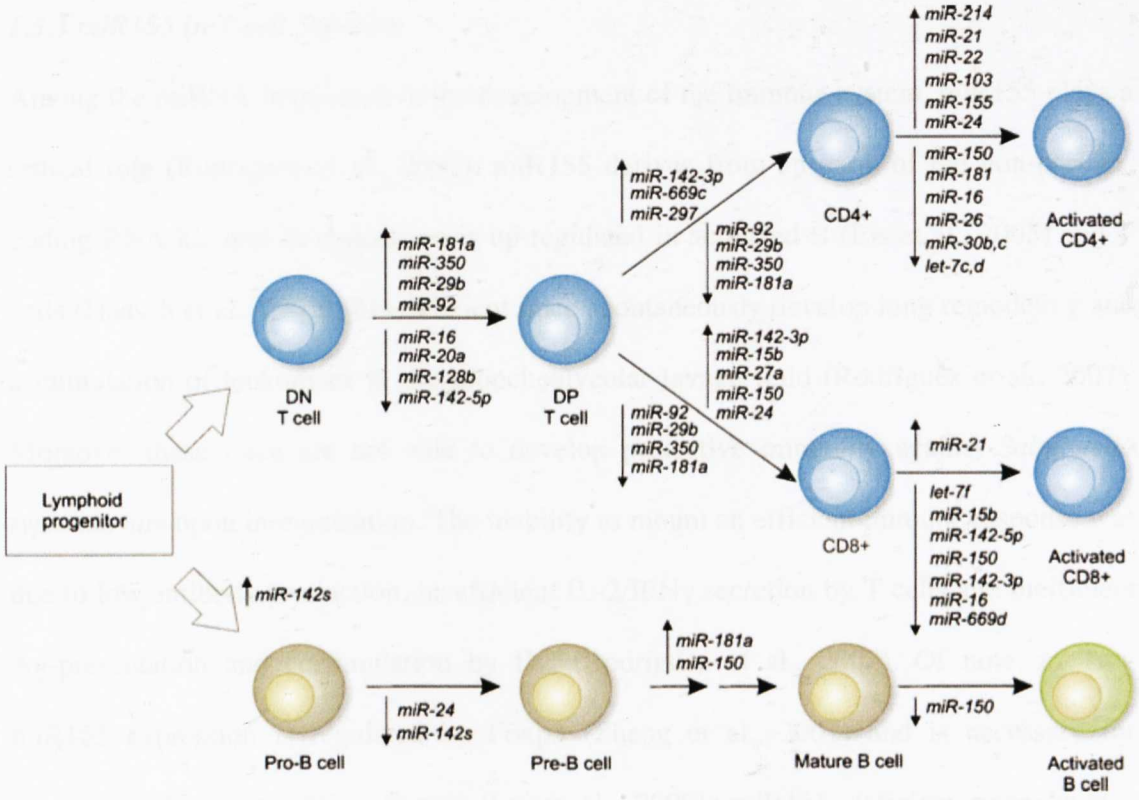


(From Kai Z.S et al., 2010)

Figure 1.7: miRNA genesis and function. Pri-miRNA, synthesized by RNA Pol II, are cleaved by Drosha into pre-miRNA and transported to the cytoplasm via Exportin 5. In the cytoplasm Dicer processes the pre-miRNA into a 22-nt long ds-RNA duplex, which contains the mature miRNA. The ds-RNA duplex is delivered to the Drosha complex; the selected mature ss-miRNA is separated form the antisense strand and associates with Argonaute. miRNA guides Argonaute to the target mRNA, thus down-modulating proteins synthesis.

1.5.2 miRNA in the immune system

In 2004 Chen and colleagues (Chen et al., 2004) were the first to demonstrate the relevance of miRNA for the development of immune cells. In particular they found that miR181 is crucial for B and T cell differentiation (Chen et al., 2004). Like miR181, other miRNA were found to be necessary for an adequate differentiation of adaptive immune cells, for instance miR150, miR155, miR142s and miR223 (Sonkoly et al., 2008). In experiments using conditional Dicer knockout mice, it was demonstrated that miRNA are necessary for the normal development of B cells (Koralov et al., 2008), T cells (Cobb et al., 2005) and NKT cells (Zhou et al., 2009a). Conditional Dicer deletion in correspondence to different stages of cell maturation causes different outcomes. For instance, abrogation of Dicer expression in immature thymocytes drastically reduces $CD4^+$ and $CD8^+$ single positive populations in thymus and in periphery. On the contrary, when Dicer is eliminated at the $CD4^+$ $CD8^+$ double positive stage, the frequency of mature cells in periphery is only 2-fold reduced. In this case Treg are the most affected T cell population, being 6-fold reduced (Cobb et al., 2006). Similarly, Dicer elimination in the earlier B cell differentiation stage almost completely blocks B cell differentiation (Koralov et al., 2008), while Dicer abrogation in later phase of B cell maturation causes an imbalance between marginal zone and follicular B cells (Belver et al., 2010). It is becoming clear that a tightly regulated miRNA expression is necessary for proper T and B cells maturation and functions (Sonkoly et al., 2008).



(From Sonkoly E. et al., 2008)

Figure 1.8: miRNA expression during T and B cell development. Several miRNA guide T and B cell differentiation, from the early lymphoid progenitor to terminally differentiated and activated cells.

1.5.3 miR155 in T cell function

Among the miRNA implicated in the development of the immune system, miR155 plays a critical role (Rodriguez et al., 2007). miR155 derives from an exon of the non-protein-coding RNA bic and its expression is up-regulated in activated B (Eis et al., 2005) and T cells (Haasch et al., 2002). Bic-deficient mice spontaneously develop lung remodeling and accumulation of leukocytes in the bronchoalveolar lavage fluid (Rodriguez et al., 2007). Moreover these mice are not able to develop protective immunity against *Salmonella typhimurium* upon immunization. The inability to mount an efficient immune response was due to low antibody production, insufficient IL-2/IFNγ secretion by T cells and inefficient Ag-presentation and costimulation by DC (Rodriguez et al., 2007). Of note, in Treg miR155 expression is regulated by Foxp3 (Zheng et al., 2007) and is necessary for sustaining Treg-competitive fitness (Lu et al., 2009). miR155 deficient mice have a consistent reduction of Treg, both in thymus and in periphery, due to an impaired proliferative capacity of Treg (Kohlhaas et al., 2009; Lu et al., 2009). Indeed, in non-lymphopenic conditions, miR155 deficient Treg display lower sensitivity to IL-2, caused by high level of SOCS1 (target of miR155), and ineffective Stat5 activation (Lu et al., 2009). On the contrary miR155 deficiency does not impact on Treg suppressive function, as shown both *in vitro* (Stahl et al., 2009) and *in vivo* experiments (Kohlhaas et al., 2009). In a model of T cell-induced colitis, the co-transfer of miR155 deficient Treg with colitogenic $CD4^{+}CD45RB^{high}$ T cells efficiently protects the recipients from colitis symptoms (Kohlhaas et al., 2009). On the contrary, modulation of miR155 in Teff strongly impacts on their functions. *In vitro* the over-expression of miR155 renders Teff resistant to the control exerted by Treg, while its down-modulation renders Teff more susceptible to Treg suppression (Stahl et al., 2009). Two different studies have demonstrated the involvement of miR155 in EAE onset (Murugaiyan et al., 2011; O'Connell et al., 2010).

EAE is an autoimmune disease caused by Th1 and Th17 inflammatory cells, and miR155 over-expression favours the differentiation of these T cell subsets, exacerbating EAE outcome (O'Connell et al., 2010). On the contrary miR155 deficient mice show delayed and milder EAE symptoms (Murugaiyan et al., 2011). In lupus-prone mice (MRL-Fas$^{lpr/lpr}$), Treg accumulate in lymphoid tissues, but display an altered phenotype ($CD62L^{-}CD69^{+}$) and are endowed with low inhibitory functions. Intriguingly, Treg isolated from these mice have low levels of Dicer, but express high amounts of miR155. In addition, the exogenous expression of miR155 in wt Treg induces the down-modulation of CD62L, mirroring the phenotype of MRL-Fas$^{lpr/lpr}$ Treg (Divekar et al., 2011). In aberrant anaplastic lymphoma kinase (ALK)-negative anaplastic large-cell lymphoma (ALCL), miR155 expression is higher than in ALK^{+}ALCL (Merkel et al., 2010). Deregulation of miR155 was observed also in Human T-cell leukemia virus type-I (HTLV-I) and adult T-cell leukemia (ATL) (Bellon et al., 2009).

All these data indicate that miR155 plays a critical role in regulating T cell homeostasis and functions, and understanding how to modulate its expression will provide great advantage for the development of new therapies for both autoimmune diseases and tumors.

2 Materials and Methods

2.1 Mice and treatments

BALB/c and C57BL/6 mice were purchased from Charles River Laboratory (Calco, Italy); $CD40^{-/-}$ and $OX40^{-/-}$ mice were provided by L. Adorini (Intercept Pharma, Perugia, Italy) and N. Killeen (UCSF), respectively. R. Furlan (San Raffaele Scientific Institute, Milan, Italy) upon agreement with A. Rudensky (New York, USA) kindly provided Foxp3-GFP mice. All these strains were backcrossed for ten generations to BALB/c. C57BL/6 CD45.1 and $Rag1^{-/-}$ mice were purchased from Jackson Laboratories. Lck_miR155 (miR155) transgenic (tg) mice (Ranganathan et al., 2012) were kindly provided by C.M. Croce (Ohio State University, Columbus, USA). miR155 tg mice have been acquired on a mixed C3HJxC57BL/6 background and were backcrossed to C57BL/6 backround. Mice were maintained under pathogen-free conditions in our animal facility and used at 8 weeks of age unless otherwise specified. Animal experiments were authorized by the Fondazione IRCCS Istituto Nazionale dei Tumori Ethical Committee for animal use and were performed in accordance to the national law (DL116/92).

CT26 is an undifferentiated colon carcinoma cell line derived from BALB/c mice; TSA is a tumor cell line derived from a spontaneous breast carcinoma on BALB/c background; MCA203 is a fibrosarcoma tumor cell line induced by 3-methylcholanthrene on C57BL/6 background, MCA38 is a colon adenocarcinoma on C57BL/6 background. B16/F10 is a melanoma tumor cell line on C57BL/6 background. Tumor cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS. 5×10^4 CT26 cells, 5×10^4 TSA cells, 5×10^5 MCA203, 5×10^4 MCA38, or 1×10^5 B16/F10 cells were inoculated s.c in the left flank of mice and tumor growth was monitored twice a week. Tumor volume was evaluated as longest diameter x (shortest diameter)^2 and expressed in cubic millimeters (mm^3). Animals were treated with intra-tumoral (i.t) injection of 50 μg of purified anti-OX40 mAb (clone

OX86, European Collection of Cell Cultures) or isotype matched control Ab –rat IgG- (mock) and were sacrificed after 24 hours for analysis. The hybridoma for anti-CD25 mAb (PC61) was kindly provided by V. Bronte (Verona University Hospital, Italy). Mice received 1 mg of PC61 once i.p.

2.2 Antibodies and flow cytometry analysis

FITC and PerCPCy5.5 anti-CD44 (IM7), FITC anti-GITR (DTA1), FITC anti-CD69 (H1.2F3), PE anti-OX40 (OX86), PE and PerCPCy5.5 anti-IL-10 (JES5-16E3), PE and *APC* anti-Foxp3 (FJK-16S), PE-Cy7 anti-CD4 (L3T4), PE anti-K^d (SF1-1.1.1), PE anti-CD25 (PC61.5), PE-Cy7 anti-CD11c (N418), *APC* anti-CD62L (Mel14), PE anti-CD80 (16-10A1), *APC* anti-CD86 (GL1), PE anti-CD8 (53-6.7), PE anti-B220 (RA3-6B2), PE anti-CD11b (MI/70), FITC anti-F4/80 (BM8), *APC* anti-CD45.2 (104), PE anti-Pd1 (J43), PE anti-IFN-γ (XMG1.1, A-B), PE anti-TNF-α (MP6-XT22, C-D), PE anti-IL-6 (MP5-20F3, E-F), PE anti-IL-17 (eBio17B7, G-H) and streptavidin-PE were from eBioscience. Biotin anti-CD40L (MR1) was from BD Pharmingen. Antibodies were used at 5 μg/ml. Surface staining was performed in 1X PBS supplemented with 2% FBS for 30 minutes on ice. Intracellular staining of Foxp3 and cytokines was performed according to manufacturer's instruction (eBioscience). Before IFNγ, TNFα, IL-6, IL-17 and IL-10 intracellular staining, cells were *in vitro* re-stimulated for 4 hours at 37°C with Phorbol Myristate Acetate (PMA) (50 ng/ml, SIGMA), Ionomycin Calcium Salt (Iono) (500 ng/ml, SIGMA) and Monensin (eBioscience) or BrefeldinA (10 μg/ml SIGMA). Flow cytometry data were acquired on a LSRFortessa (Becton Dickinson) and analyzed with FlowJo software (version 8.8.6, Tree Star Inc.)

2.3 pStat5 staining

Purified wt and OX40$^{-/-}$ Treg were stimulated with increasing doses (0, 1, 10, 100, 100 IU/ml) of recombinant (r) IL-2 (Proleukin) for different time intervals (1, 5, 10 minutes). Treg were stained with AlexaFluor-488 anti-Stat5 pY694 (clone 47) according to the manufacturer's instructions (BD Bioscience).

2.4 AnnexinV and BrdU staining

5-weeks old wt and OX40$^{-/-}$ mice were thymectomized by the suction method. After 3 weeks lymph nodes were collected from each mouse. Before collection, BrdU was administered according to the following schedule. On day 0 mice were i.p. injeceted once with 1 mg of BrdU (Sigma-Aldrich). On days 1, 2 and 3 BrdU was provided in drinking water at 0.8 mg/ml. On day 4 lymph nodes were collected and stained with AnnexinV Apoptosis Detection Kit according to manufacture's instruction (eBioscience). For BrdU analysis cells were treated with Fix/Perm buffer (eBioscience) and then stained with the BrdU Flow kit (BD Pharmingen). BrdU (BD Pharmingen) and Foxp3 (eBioscience) antibodies were simultaneously added to permeabilized cells.

2.5 Bone marrow transplantation

Female (BALB/c x C57BL/6) F1 mice were irradiated at 600+600 Rad with an interval of 3 hours and received 10^7 bone marrow cells from IL-10-GFP C57BL/6 female mice (bone marrows kindly provided by Giorgio Trinchieri, NCI, Frederick). 8 weeks after transplantation, correct reconstitution was checked by flow cytometry: peripheral blood cells were stained with PE-conjugated anti-K^d, PE-Cy7-conjugated anti-CD4 and *APC*-conjugated anti-Foxp3. Optimally transplanted mice were subcutaneously inoculated with CT26 cells and treated with OX86 or isotype matched control Ab. After 24 hours tumors

were collected and GFP fluorescence was evaluated in $CD4^+CD25^{high}$ cells without any restimulation. In this experiment Treg could not be identified as $CD4^+Foxp3^+$ cells because the fixation/permeabilization step, required for Foxp3 intracellular staining, induced the loss of GFP expression.

2.6 Isolation of tumor-associated macrophages (TAM)

BALB/c mice were subcutaneously inoculated with CT26 tumor cells and subsequently treated with OX86 or isotype matched control Ab. 24 hours upon treatment tumors were collected and disaggregated by 0.125% (wt/vol) trypsin (Lonza) for 40 minutes at 37°C. Disaggregated cells were then washed in medium and seeded in 140-mm Petri dishes for 2 hours at 37°C. Non-adherent cells were eliminated while adherent cells, which are mainly macrophages, were harvested using EDTA 2 mM. To detect IL-10 secretion macrophages were restimulated for 4 hours at 37°C with PMA 50 ng/m (SIGMA), Iono (500 ng/ml, SIGMA) and BrefeldinA (SIGMA). Cells were subsequently stained for the surface markers FITC anti-F4/80, PE anti-CD11b and *APC* anti-CD45.2 to specifically identify macrophages. IL-10 was detected by intracellular staining according to manufacturer's instruction (eBioscience) using PerCP-Cy5.5 anti-IL-10 (JES5-16E3).

2.7 Migration of dendritic cells from the tumor to the dLN

BALB/c and $CD40^{-/-}$ CT26 tumor-bearing mice were intratumorally injected with 50 μg of OX86 or isotype matched control Ab, plus $4x10^7$ FITC-conjugated latex micro-spheres of 1 μm in diameter (Polysciences), which could be uptaken by DC. After 24 hours, tumor dLN were collected, mechanically and enzymatically disaggregated (by incubation for 30 min at 37°C with 400 U/ml of collagenase D). Migrated DC were identified as $FITC^+$ CD11c PE-

$Cy7^+$ cells by FACS analysis. Absolute number of migrated DC was evaluated for each sample.

2.8 In vitro differentiation of bone marrow-derived dendritic cells (BM-DC)

BM-DC were differentiated from BM precursors isolated from femurs and tibias of BALB/c and $CD40^{-/-}$ mice. Cells were cultured for 10 days in IMDM with 10% FBS supplemented with conditioned medium from a murine fibroblast cell line engineered to express mGM-CSF (corresponding to 20 ng/ml of rGM-CSF). The differentiation state of DC was checked according to CD11c expression, evaluated by flow cytometry analysis.

2.9 Induction of Tem via BM-DC immunization

BM-DC differentiated from wt BM precursors were in vitro activated o.n. with LPS (100 ng/ml). BALB/c mice were treated with 2 consecutive injections of 10^6 activated BM-DC. After 4 weeks, Tem were sorted from total splenocytes as $CD4^+CD44^{high}CD62L^{low}$ cells. OX40 expression on Tem, evaluated by FACS, was analyzed at different time points upon *in vitro* activation with 1 µg/ml of αCD3 (145-2c11, eBioscience) and 0.5 µg/ml of αCD28 (37.51, BD Pharmingen).

2.10 Co-culture of BM-DC and Tem

Tumor-infiltrating lymphocytes were enriched by ficoll gradient from single-cell suspensions of mechanically disaggregated tumors 24 hours after OX86 or isotype matched Ab treatment. $CD4^+CD44^{high}CD62L^{low}$ Tem were sorted using a FACSaria (Becton Dickinson) from TIL pooled from different mice which have received the same treatment. OX86 treated or control Tem were co-cultured with wt or $CD40^{-/-}$ BM-DC for 24

hours at 1:1 ratio. BM-DC activation was analyzed by flow cytometry according to CD11c, CD80 and CD86 expression level, compared to BM-DC cultured alone.

2.11 Treg sorting and gene expression profiling

Treg were sorted from the spleen of Foxp3-GFP mice as $CD4^+GFP^+CD8^-B220^-CD11b^-$ cells (FACSAria, Becton Dickinson). Purity after sorting assessed around 98%. Pooled Treg were activated overnight with coated anti-CD3 (1 μg/ml) plus OX86 or isotype control matched Ab -rat IgG- (10 μg/ml). RNA was purified using mirVana Kit (Ambion), and checked for integrity and purity by Agilent Bioanalyzer. Each sample was analyzed in duplicate.

RNA (0.2 μg) was reverse transcribed, labeled with biotin and amplified using the Illumina RNA TotalPrep amplification kit (Ambion). Biotinylated sample (1 μg) was hybridized at 58°C overnight to an expression Bead Chip MouseRef_8_v2.0 array (Illumina). Array chips were washed, stained with 1 μg/ml Cy3-streptavidin (GE Healthcare Europe GmbH) and scanned with an Illumina BeadArray Reader (Illumina). Data were analyzed using the BeadStudio Gene Expression Module v3 (Illumina). Intensity values were quality checked, and the data set was normalized using a cubic spline algorithm. A detection p value <0.05 was set as a cut-off to filter reliable genes. All array data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE32373.

Class comparison analysis to identify differentially expressed genes between Treg activated with OX86 or isotype control was performed using the GenePattern Software (Broad Institute-MIT).

2.12 IRF1 Real Time RT_PCR

Foxp3-GFP mice were subcutaneously inoculated with CT26 and intratumorally injected with OX86 or rat IgG. After 24 hours, Treg were sorted from TIL according to GFP expression. Control Treg were sorted from spleens of Foxp3-GFP tumor-free mice. RNA was extracted according to the manufacturer's instructions (RNeasy MICROKIT, Qiagen) and reverse transcribed using High-Capacity® cDNA Reverse Transcription Kits (Applied Biosystem). Real time RT_PCR was performed on 7900 HT (Applied Biosystem), using TaqMan® Fast Universal PCR masterMix (Applied Biosystem). Assays (Applied Biosystem) and samples were normalized over HPRT1 expression. Data were analyzed using the comparative Ct method (Schmittgen and Livak, 2008). Amplicon length for IRF1 is 66 nucleotides, while for HPRT1 is 81 nucleotides.

2.13 miR155 Real Time RT_PCR

Purified wt and $OX40^{-/-}$ Treg were lysed and RNA was extracted using mirVana miRNA isolation kit according to the manufacturer's instructions (Ambion). 50 nanograms of RNA were reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem). Real time RT_PCR was performed on 7900 HT (Applied Biosystem), using TaqMan® Fast Universal PCR masterMix (Applied Biosystem), according to TaqMan MicroRNA Assays protocol (Applied Biosystem). Normalization was done according to RNU6 expression. Data were analysed using the comparative Ct method (Schmittgen and Livak, 2008).

2.14 Treg isolation and transfer into $Rag1^{-/-}$ mice

Total splenocytes from CD45.1, wt CD45.2 and $OX40^{-/-}$ CD45.2 mice were enriched of $CD4^+$ T cells by passing on a nylon wool column (Kisker). Using αCD8 and αB220

magnetic microbeads $CD8^+$ T cells and B cells were eliminated by passing into a magnetic separator (Miltenyi Biotec). $CD4^+CD25^+$ regulatory T cells were purified using the $CD25^+$ T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. $Rag1^{-/-}$ mice were i.v injected with 4 x 10^5 CD45.1 Treg, mixed 1:1 with wt or $OX40^{-/-}$ CD45.2 Treg.

2.15 Induction and analysis of colitis

$CD4^+$ T cells were purified form the spleen of CD45.1 mice by negative magnetic separation according to manufacturer's instructions (Miltenyi Biotec). $CD4^+$ T cells were stained with FITC-anti CD45RB and $CD45RB^{high}$ naïve T cells were purified on a FACSAria (Beckton Dickinson). $Rag1^{-/-}$ mice were i.p injected with a total of 5 x 10^5 sorted cells. Colitis onset was evaluated as weight loss and diarrhea. At day 13, at the onset of the symptoms, mice were left untreated (control mice) or received 1.4 x 10^6 purified wt or $OX40^{-/-}$ CD45.2 Treg. Mice weight was monitored every 2 days. Serum, for TNF-α evaluation, was collected on day 37 after colitis induction and its concentration was evaluated by ELISA, according to manufacturer's instruction (eBioscience). For histological analysis hematoxylin-eosin (H&E) staining was done on paraffine-embebbed colon sections. Severity of the disease was evaluated according to a four-grade semi-quantitative scoring system.

2.16 SOCS1 western blot

Treg were purified from the spleen of wt and $OX40^{-/-}$ mice using magnetic microbeads (Miltenyi Biotec) as previously described. As control cells naïve and activated Teff purified form both wt and $OX40^{-/-}$ were used. Activated Teff were obtained by stimulating *in vitro* naïve Teff for 3 days with coated αCD3 (1 μg/ml, 145-2c11, eBioscience).

Cells were lysed using a chilled lysis buffer (50nM Hepes, pH7.0, 0.1% NP-40, 250 nM NaCl, 1X protease inhibitor cocktail). Proteins were separated from cell debris by centrifugation at 14000 rpm for 20 minutes at 4°C. Solubilized proteins were separated by SDS-PAGE and transferred to a PVD membrane. SOCS1 antibody (4H1) was from Invitrogen, β-actin (A2066) from Sigma. Densitometric analysis was done using the software NIH Image J.

2.17 In vitro suppression assay

$CD4^+CD25^+$ Treg and $CD4^+CD25^-$ Teff were purified from the spleen of miR155 tg mice and wt littermates using magnetic beads (Miltenyi Biotec). Teff were labelled with 5μM 5-(and-6)-Carboxyfluorescein Diacetated Succinimidyl Ester (CFSE) (Invitrogen) in PBS plus 5% FBS for 15 minutes at 37°C and then washed twice in PBS. In a 96-well round bottom plate Teff were seeded 1 x 10^5/well with 7,5 x 10^4 accessory cells (AC, consisting of irradiated splenocytes). For stimulation 1 μg/ml αCD3 (145-2c11, eBioscience) was added. Teff and Treg were co-cultured in a Teff:Treg increasing ratio (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64). Teff proliferation was evaluated 72 hours after co-culture as CFSE dilution by flow cytometry.

2.18 Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, Inc). Results are expressed as means ± SEM or mean ± SD. Two-tailed student's *t*-test was used to analyse the data. Differences were considered significant at $p<0.05$ ($*p<0.05$, $**p<0.01$, $***p<0.005$ by two-tailed Student's t test).

3 Scope of the study

The awareness that the immune system plays a key role in regulating tumor onset has addressed the research toward the development of new anti-tumor therapies based on the modulation of immune cells. The costimulatory molecule OX40 seems to be a suitable tool to achieve this aim, thanks to its double ability of suppressing Treg and boosting Teff, which are pro-tumoral and anti-tumoral immune cells, respectively. OX86, an OX40 agonist mAb, gave satisfactory results, inducing the rejection of different mouse tumor models. The scope of this study is to describe how the modulation of OX40 affects Treg and Teff functions, and which are the molecular mechanisms at the base of these processes. The discovery that the constitutive expression of OX40 by Treg is necessary for assuring their adequate sensitivity to IL-2 leads to the identification of miR155 as a key player for the regulation of Treg functions. Thank to a transgenic mouse model, in which miR155 is specifically over-expressed in T cells, the role of miR155 in Treg biology is deeply investigated in this study.

4 Results

4.1 Tumor-associated Treg highly express OX40

Treg accumulate in the tumor nodules after being recruited from periphery in a chemokine dependent manner (CCL22-CCR4) (Curiel et al., 2004; Gobert et al., 2009; Mailloux et al., 2010). In addition $CD4^+Foxp3^-$ cells locally convert into regulatory cells as a consequence of the strong immunosuppressive stimuli concentrated in the tumor mass. Treg inhibit target cells using several strategies, which include cell-cell contact mechanisms (for instance CTLA4, LAG3), and the release of soluble immune-inhibitory mediators (for instance IL-10, IL-35, TGFβ) (Vignali et al., 2008). It was demonstrated that the intra-tumoral injection of OX86, an agonist of OX40, lowers the suppression exerted by Treg and also prevents the generation of new Treg from non-regulatory $CD4^+$ TIL (Piconese et al., 2008). Treg constitutively express OX40, but its expression level may change when Treg are challenged by different external stimuli, thus determining the strength of the response to OX86. For this reason OX40 expression was evaluated on Treg isolated from spleen, draining lymph node (dLN) and tumor nodules. BALB/c mice were subcutaneously inoculated with the transplantable CT26 colon carcinoma cell line. When tumors were about 8x8 mm in size, mice were sacrificed and tumor, dLN and spleen of each mouse were collected. Treg were identified as $CD4^+Foxp3^+$ cells, OX40 expression was evaluated by flow cytometry as mean fluorescent intensity (MFI). Tumor-infiltrating Treg express OX40 at higher level compared to Treg localized in the dLN and spleen (Fig 4.1). The control for OX40 staining was done on Treg isolated from tumor, dLN and spleen collected from CT26 bearing $OX40^{-/-}$ mice. These data confirmed that OX40 expression is modulated on Treg according to the microenvironmental features, and also that the intra-tumoral OX86 injection directly impacts on Treg biology.

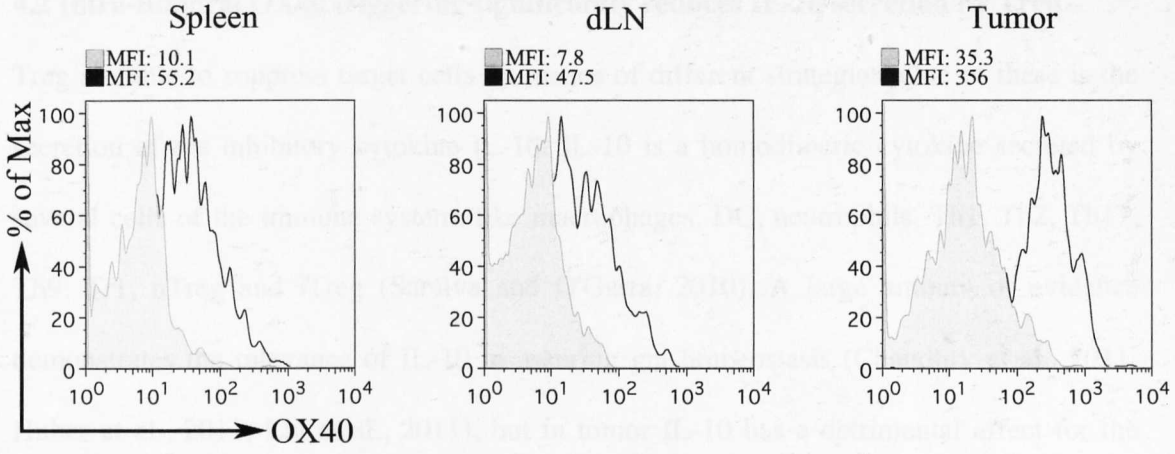


Figure 4.1: OX40 expression on Treg in spleen, draining lymph node and tumor. BALB/c and OX40$^{-/-}$ mice were subcutaneously injected with CT26 tumor cells. 8x8 mm tumors, dLN and spleens were collected from each mouse. MFI of OX40 was evaluated on wt (open histogram) and OX40$^{-/-}$ (grey histogram) Treg isolated from each anatomical district. Tumor-associated Treg expressed OX40 at higher level than Treg isolated from dLN and spleen. MFI: mean fluorescent intensity; dLN: draining lymph node.

4.2 Intra-tumoral OX40 triggering significantly reduces IL-10 secretion by Treg

Treg are able to suppress target cells by means of different strategies. One of these is the secretion of the inhibitory cytokine IL-10. IL-10 is a homodimeric cytokine secreted by several cells of the immune system, like macrophages, DC, neutrophils, Th1, Th2, Th17, Th9, Tr1, nTreg and iTreg (Saraiva and O'Garra, 2010). A large amount of evidence demonstrates the relevance of IL-10 in assuring gut homeostasis (Chaudhry et al., 2011; Huber et al., 2011; Liu et al., 2011), but in tumor IL-10 has a detrimental effect for the host, keeping immune cells in an inactive/immature state and favouring tumor growth (Dercamp et al., 2005). In this regard it was checked whether OX86 affects IL-10 production by Treg. CT26 tumor-bearing mice were treated with OX86 or the isotype matched control Ab (rat IgG mock) and 24 hours later IL-10 secretion by Treg infiltrating the tumor or the dLN was evaluated *ex vivo* by cytokine intracellular staining (ICS). Before the staining Treg were restimulated with PMA, IONO and BFA. In the presence of OX86 or the control Ab, low levels of IL-10 were produced by Treg in the dLN (Fig 4.2 A-B). Conversely, in tumor microenvironment, about 40% of Treg spontaneously produced IL-10 (Fig 4.2 C-D, mock), but OX40 engagement significantly decreased their ability to secrete this inhibitory cytokine (Fig 4.2 C-D, OX86). Similar results were obtained also in mice bearing TSA mammary carcinoma (Fig 4.2 E-H) or MCA203 fibrosarcoma (data not shown). These data confirmed that OX86 could antagonize the inhibitory functions of Treg directly *in vivo*. Moreover these results suggest that Treg can be oriented to use specific suppressive mechanisms according to the peculiar characteristic of the microenvironment, since only tumor-infiltrating Treg, but not dLN-Treg, secrete IL-10.

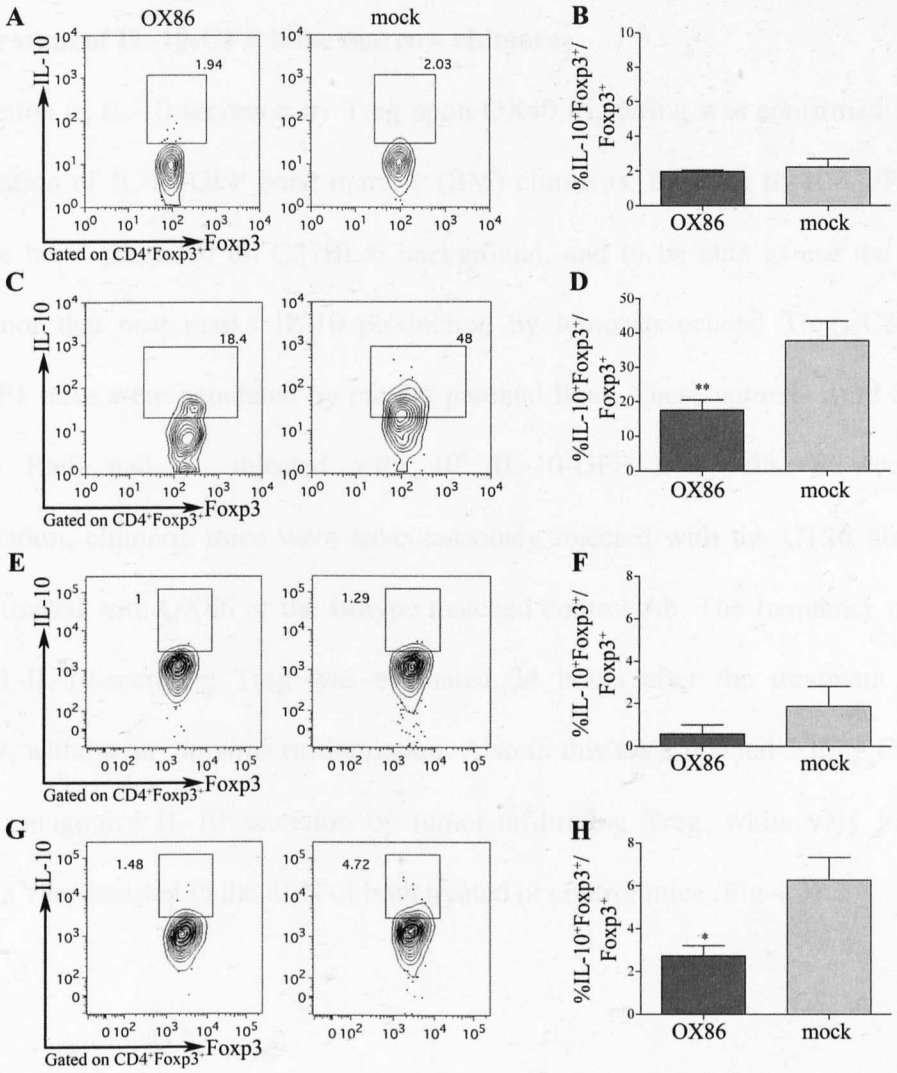


Figure 4.2: OX86 reduces IL-10 secretion by tumor infiltrating Treg. (A) Representative plots and (B) frequency of IL-10-producing Treg cells in the CT26-dLN 24 hours after intra-tumoral injection of OX86 or isotype matched control Ab (mock). (C) Representative plots and (D) frequency of IL-10-producing Treg cells in CT26 tumors 24 hours after OX86 or isotype matched control Ab (mock) treatment. (E) Representative plots and (F) frequency of IL-10-producing Treg in TSA-dLN in the presence or absence (mock) of OX86. (G) Representative plots and (H) frequency of IL-10-producing Treg in TSA tumor nodules upon OX86 or mock treatment. Data are presented as means ± SEM of n=5 mice per group and are representative of two different experiments. **p<0.01, ***p<0.005, two-tailed Student's t-test.

4.3 Generation of IL-10-GFP bone marrow chimeras

The reduction of IL-10 secretion by Treg upon OX40 triggering was confirmed also with the generation of IL-10-GFP bone marrow (BM) chimeras. Because IL-10-GFP reporter mice have been generated on C57BL/6 background, and to be able to use the BALB/c CT26 tumor that best marks IL-10 production by tumor-associated Treg, C57BL/6 x BALB/c F1 mice were generated by mating parental lines. These animals were irradiated (600+600 Rad) and i.v injected with 10^6 IL-10-GFP BM cells. 8 weeks after transplantation, chimeric mice were subcutaneously injected with the CT26 tumor cells and then treated with OX86 or the isotype matched control Ab. The frequency of tumor-associated IL-10-secreting Treg was evaluated 24 hours after the treatment by flow cytometry, without any *in vitro* restimulation. Also in this experimental setting OX86 was found to antagonize IL-10 secretion by tumor-infiltrating Treg, while very low IL-10 production was detected in the dLN of both treated or control mice (Fig 4.3).

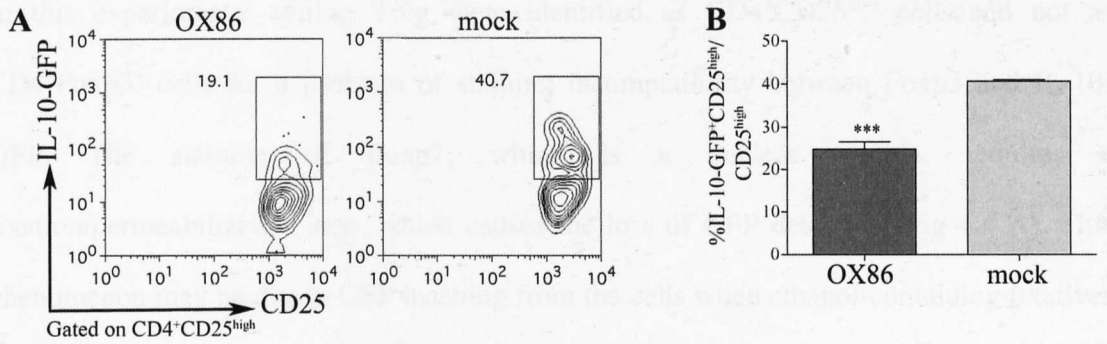


Figure 4.3: reduction of IL-10-GFP secreting Treg in tumor-bearing BM chimeras upon OX86 treatment. IL-10-GFP bone marrow chimeras were subcutaneously inoculated with CT26 tumor cells. When tumor nodule where about 3x3 mm in size were treated with OX86 or isotype matched control Ab (mock). Secretion of IL-10-GFP by $CD4^+CD25^{high}$ Treg was evaluated 24 hours after treatment. (A) Representative plots and (B) frequency of IL-10-GFP-positive Treg cells in the CT26 nodules 24 hours after intra-tumoral injection of OX86 or isotype matched control Ab (mock). Data are presented as means ± SEM of n=5 mice per group and are representative of two independent experiments. ***$p<0.005$, two-tailed Student's t-test.

In this experimental setting Treg were identified as $CD4^+CD25^{high}$ cells and not as $CD4^+Foxp3^+$ cells for a problem of staining incompatibility between Foxp3 and IL-10-GFP. The staining of Foxp3, which is a nuclear protein, requires a fixation/permeabilization step, which causes the loss of GFP detection (Fig 4.4 A). This phenomenon may be due to GFP leaching from the cells when ethanol-containing fixatives are used (Kalejta et al., 1997). To be sure that $CD4^+CD25^{high}$IL-10-GFP^+ cells were Treg, Foxp3 expression was evaluated in $CD4^+$ T lymphocytes expressing CD25 at different levels (low, medium, high). While $CD4^+$ gated on $CD25^{low}$ and $CD25^{medium}$ T cells were mainly Foxp3 negative, the majority of $CD4^+CD25^{high}$ cells were $Foxp3^+$ (Fig 4.4 B). These data further supported previous results and confirmed that Treg are impaired in their ability to secrete IL-10 upon OX40 engagement.

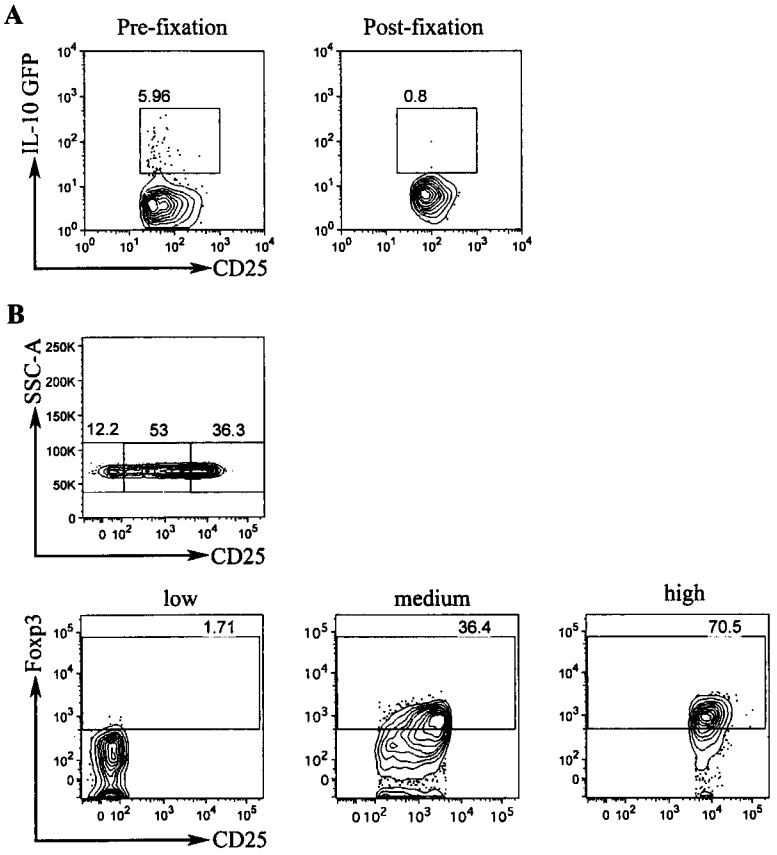


Figure 4.4: Fixation/permabilization leads to the loss of GFP detection. A) TIL were enriched from CT26 tumors grown in IL-10-GFP > C57BL/6 x BALB/c BM chimeras and surface staining for CD4 and CD25 was performed. Then, cells were fixed with Foxp3 Fixation/ Permeabilization buffer. GFP signal was analysed by flow cytometry in gated $CD4^+$ TIL before and after fixation. Representative dot plots are shown. B) TIL from CT26 tumors were stained for CD4, CD25 and Foxp3. $CD4^+$ gated T cells were divided in three subsets according to CD25 expression ($CD25^{low}$, $CD25^{medium}$ and $CD25^{high}$). The majority of $CD25^{high}$ $CD4^+$ T cells in the tumor were composed of Foxp3-positive T cells. TIL: tumor infiltrating lymphocytes, BM: bone marrow.

4.4 OX40 triggering on Treg leads to IRF1 down-regulation

To better understand how OX40 triggering modifies Treg biology an analysis of the trascriptome of naïve Treg was performed. Treg were sorted from the spleens of Foxp3-GFP tumor free (*TF*) mice as $CD4^+GFP^+CD8^-B220^-CD11b^-$ cells and stimulated *in vitro* over night (o.n.) with anti-CD3 coated-wells plus OX86 or isotype matched control Ab (rat IgG). The gene expression analysis showed that 9 genes were up-regulated and 12 down-regulated more than 1.3-fold by OX40 stimulation (Table 4.1). Among the down-modulated targets, there were two probes belonging to the interferon regulatory factor 1 (IRF1) mRNA, a transcription factor known to promote IL-10 expression in human cells (Ziegler-Heitbrock et al., 2003) (Fig 4.5 A). To validate this data also in tumor-associated Treg, Foxp3-GFP mice were subcutaneously inoculated with CT26 cells and then tumor nodules were treated with OX86 or isotype matched control Ab (mock). Treg were sorted from pooled tumors 24 hours after treatment, according to GFP expression. Sorted cells were lysed and IRF1 expression was evaluated by quantitative real time RT_PCR. Control Treg were sorted from spleens of *TF* Foxp3-GFP mice. IRF1 transcription in tumor-infiltrating Treg was about 4-fold higher than in splenic Treg from tumor-free mice. Intratumoral OX86 treatment produced a 40% reduction in IRF1 expression by tumor-infiltrating Treg (Fig 4.5 B). The expression of IRF1 in the different samples mirrored the different amount of Treg-derived IL-10 as evaluated by FACS analysis (Fig 4.2). These data, together with gene expression data, indicate that the effect of OX40 triggering on IRF1 expression is Treg-intrinsic and that OX40 stimulation modulates IRF1 expression *in vivo* in tumor-infiltrating Treg.

The binding of IRF1 to IL-10 promoter was previously demonstrated only in human cells; to confirm this interaction in the mouse system, we performed a computational analysis of the mouse IL-10 promoter with the web tool TESS. A putative IRF1 binding site (BS) of 6

nucleotides (AAGTGA) was found between -1470/-1476 nt in the IL-10 promoter region. To reinforce this data, we investigated if the same IRF1 BS was present also in the promoter sequence of two other genes known to be regulated by IRF1: VCAM-1 and Viperin (Stirnweiss et al., 2010; Warfel and D'Agnillo, 2008). The presence of this IRF1 BS was confirmed also in the promoter of these two additional target genes by TESS analysis (Fig 4.5 C). Although other experiments are required to demonstrate that IRF1 directly binds to IL-10 promoter, these data display that Treg reduce IL-10 secretion upon OX86 treatment as a consequence of IRF1 down-modulation.

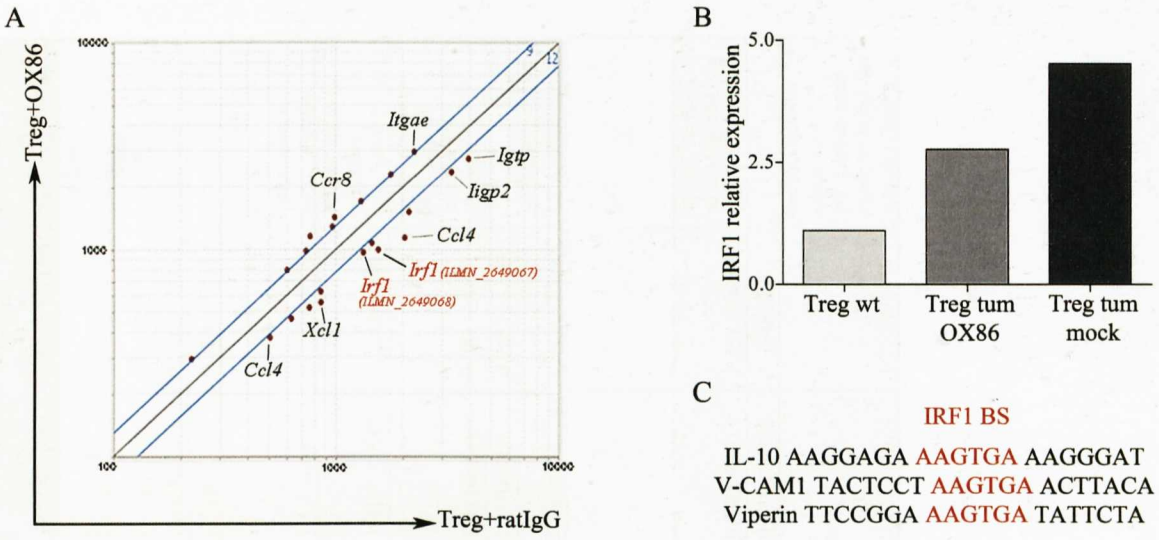


Figure 4.5: OX86 modulates IRF1 expression in Treg. A) Mean expression plot representing genes differentially expressed by Treg activated with OX86 versus isotype matched control Ab (rat IgG). External blue lines in the graph represent a FC of 1.3. Probes for genes of interest are shown. B) Foxp3-GFP Treg were sorted from tumors according to GFP expression, 24 hours after OX86 or isotype matched control Ab (mock) injection. Control Treg were sorted from spleens of tumor-free Foxp3-GFP mice. IRF1 expression was evaluated by quantitative real-time PCR. Data are shown as mean of n=3-5 pooled spleens or n=5-6 pooled tumors per group and are representative of two independent experiments. C) IRF1 BS in IL-10, VCAM-1 and Viperin promoter sequences. Analysis was performed using the web tool TESS. FC: fold change, BS: binding site.

Table 4.1: gene expression analysis in Treg stimulated or not with OX86

Name	Description	TregIg no1	TregIg no2	TregOX86 no1	TregOX86no2	av Ig	av OX86	FC OX86/Ig	FC Ig/OX86	Name
ILMN_2611000	Il1r2	619.9893	908.9711	992.4017	1311.062	764.4802	1151.73185	1.5065555	0.663765789	interleukin 1 receptor, type II
ILMN_2724465	Ccr8	903.6345	1080.221	1502.726	1360.163	991.92775	1431.4445	1.443093512	0.692955787	chemokine (C-C motif) receptor 8
ILMN_3009572	Txn1	846.4689	624.8725	965.1298	1001.885	735.6707	983.5074	1.336885375	0.748007285	thioredoxin 1
ILMN_2865016	Cd83	998.4727	929.2532	1272.064	1283.363	963.86295	1277.7135	1.325617402	0.754365474	CD83 antigen
ILMN_2966991	Giyd2	244.67	204.1759	320.4615	271.6561	224.42295	296.0588	1.319200198	0.758035059	SLX1 structure-specific endonuclease subunit homolog B
ILMN_1217629	Itgae	1965.691	2540.641	2346.927	3591.394	2253.166	2969.1605	1.317772636	0.758856249	integrin alpha E, epithelial-associated (CD103)
ILMN_1249378	Bhlhb2	1678.277	1843.505	2437.754	2180.945	1760.891	2309.3495	1.311466468	0.762505199	basic helix-loop-helix family, member e40 (Dec1)
ILMN_2621596	LOC621823	1277.368	1319.034	1669.18	1721.281	1298.201	1695.2305	1.30583053	0.765796156	
ILMN_1250011	Tob1	734.0581	477.2617	825.0547	754.0446	605.6599	789.54965	1.30361883	0.767095394	transducer of ErbB-2.1
ILMN_1224472	Ccl4	419.7827	591.123	332.072	416.0875	505.45285	374.07975	0.740088319	1.351190087	chemokine (C-C motif) ligand 4 (MIP1b)
ILMN_3162205	A130090K04Rik	836.4427	869.9786	595.9619	663.5125	853.21065	629.7372	0.738079395	1.354867792	
ILMN_2755008	Nfkbiz	1409.79	1506.213	966.9918	1184.393	1458.0015	1075.6924	0.737785524	1.355407457	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

ILMN_2677772	Srxn1	551.8477	712.5577	388.5158	539.3362	632.2027	463.926	0.733824769	1.36272315	sulfiredoxin 1 homolog
ILMN_2649068	Irf1	1055.755	1603.18	882.9554	1044.196	1329.4675	963.5757	0.724783193	1.379722942	interferon regulatory factor 1
ILMN_1259564	Iigp2	2830.084	3817.298	2118.951	2648.352	3323.691	2383.6515	0.717170008	1.394369521	immunity-related GTPase family M member 2
ILMN_2846865	Actb	739.7339	3537.514	1174.159	1860.577	2138.6239 5	1517.368	0.70950669	1.40942998	actin, beta
ILMN_2593554	Igtp	3105.861	4836.393	2294.609	3208.363	3971.127	2751.486	0.692872829	1.443266293	interferon gamma induced GTPase
ILMN_1228328	Pou2af1	720.905	804.1101	501.5758	549.8626	762.50755	525.7192	0.689460976	1.450408412	POU domain, class 2, associating factor 1
ILMN_2948552	Xcl1	695.1525	1026.342	515.9908	591.123	860.74725	553.5569	0.643112017	1.554939068	chemokine (C motif) ligand 1
ILMN_2649067	Irf1	1251.549	1863.253	932.0236	1068.857	1557.401	1000.4403	0.642378103	1.556715578	interferon regulatory factor 1
ILMN_1223257	Ccl4	1684.059	2392.419	974.0643	1298.797	2038.239	1136.43065	0.557555149	1.793544551	chemokine (C-C motif) ligand 4 (MIP1b)

4.5 OX86 does not affect IL-10 secretion by tumor-associated macrophages

In CT26 nodules tumor-associated macrophages (TAM) represent the most abundant immune population and these cells are known to secrete large amounts of IL-10. Although OX40 expression was not observed on TAM, OX86 may decrease their frequency (Gough et al., 2008) or indirectly affects their ability to release IL-10. To explore this hypothesis, TAM isolated from the tumor mass upon OX86 injection were restimulated *in vitro* with PMA, IONO and BFA or with BFA alone, for 4 hours at 37°C. IL-10 production was evaluated by ICS. As shown in the graph (Fig 4.6 A-B), there was no modulation in IL-10 production in the presence or absence of OX86, confirming that OX86 does not modify TAM functions either directly or indirectly.

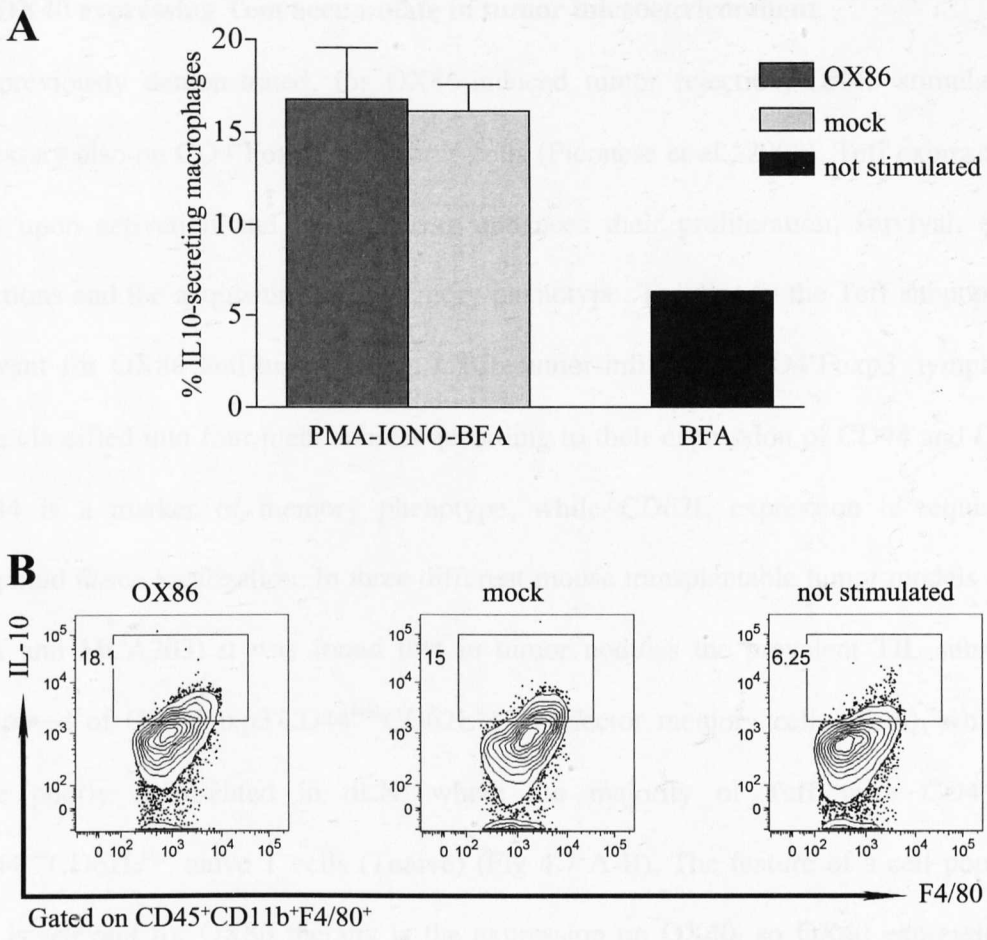


Figure 4.6: OX86 does not alter IL-10 secretion by tumor-associated macrophages. CT26 tumor cells were subcutaneously injected into BALB/c mice. When tumor size was 3x3 mm, OX86 or isotype matched control Ab (mock) was given intratumorally. After 24 hours, tumors were collected, TAM isolated by adhesion to cell culture dishes and restimulated with PMA, IONO and BFA or with BFA alone. After surface staining for CD45, CD11b and F4/80, ICS for IL-10 was performed. Percentages of IL-10$^+$ cells (A) and representative flow cytometry plots (B) in gated CD45$^+$CD11b$^+$F4/80$^+$ cells are shown. Bars represent means ± SEM. Data are representative of two independent experiments with 3 mice pour group. TAM: tumor-associated macrophages, ICS: intra-cellular staining.

4.6 OX40 expressing Tem accumulate in tumor microenvironment

As previously demonstrated, for OX86-induced tumor rejection, OX40 stimulation is necessary also on $CD4^+Foxp3^-$ effector T cells (Piconese et al., 2008). Teff express OX40 only upon activation and its triggering enhances their proliferation, survival, effector functions and the acquisition of a memory phenotype. To identify the Teff subpopulation relevant for OX86 anti-tumor effect, CT26 tumor-infiltrating $CD4^+Foxp3^-$ lymphocytes were classified into four main subsets according to their expression of CD44 and CD62L. CD44 is a marker of memory phenotype, while CD62L expression is required for lymphoid tissue localization. In three different mouse transplantable tumor models (CT26, TSA and MCA203) it was found that in tumor nodules the prevalent TIL subset was composed of $CD4^+Foxp3^-CD44^{high}CD62L^{low}$ T effector memory cells (Tem), while they were poorly represented in dLN, where the majority of Teff were $CD4^+Foxp3^-$ $CD44^{low}CD62L^{high}$ naive T cells (Tnaive) (Fig 4.7 A-B). The feature of a cell population that is relevant for OX86 therapy is the expression on OX40, so OX40 expression was checked on tumor-infiltrating Tem. Notably it was found that Tem brightly expressed OX40, although at lower levels than tumor-associated Treg. This evidence suggested that the intratumoral OX86 injection could directly target Tem and exerts its anti-tumoral effect through this T cell population (Fig 4.7 C).

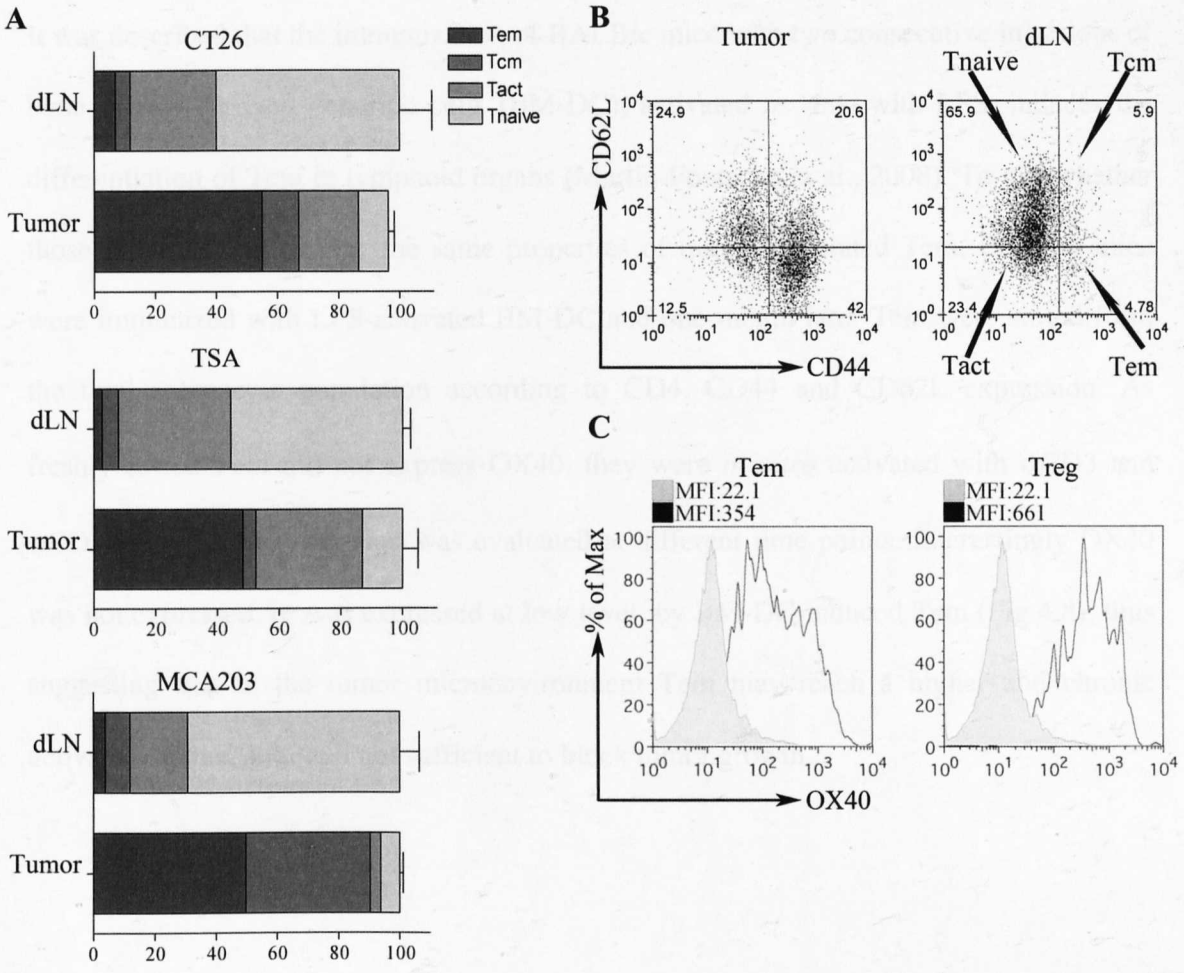


Figure 4.7: OX40-expressing Tem accumulate at tumor site. BALB/C mice were subcutaneously inoculated with CT26, TSA and MCA203 tumor cells. Tumor nodules and dLN were collected from each mouse and infiltrating lymphocytes were classified according to CD44 and CD62L expression. A) Frequency of T cell subsets in tumor and dLN collected from mice bearing CT26, TSA and MCA203 tumors. (B) Representative plots of Tem, Tcm, Tact and Tnaive in CT26 tumor and dLN. C) MFI of OX40 on CT26 tumor-infiltrating Tem and Treg cells. Filled grey histogram, isotype control. Tem: T effector memory T cells; Tcm: central memory T cells; Tact: recently activated T cells; MFI: mean fluorescent intensity. Data are presented as mean ± SEM of n=5-6 mice per group and are representative of three independent experiments.

4.7 Tem induced by BM-DC immunization do not express OX40

It was described that the immunization of BALB/c mice with two consecutive injections of bone marrow-derived dendritic cells (BM-DC), activated *in vitro* with LPS, induces the differentiation of Tem in lymphoid organs (Martin-Fontecha et al., 2008). To test whether those induced Tem display the same properties of tumor-associated Tem, BALB/c mice were immunized with LPS-activated BM-DC and one month later Tem were sorted from the total splenocyte population according to CD4, CD44 and CD62L expression. As freshly sorted Tem did not express OX40, they were *in vitro* activated with αCD3 and αCD28 and OX40 expression was evaluated at different time points. Interestingly OX40 was not expressed, or was expressed at low level, by BM-DC-induced Tem (Fig 4.8), thus suggesting that in the tumor microenvironment Tem may reach a higher and chronic activation status, although not sufficient to block tumor growth.

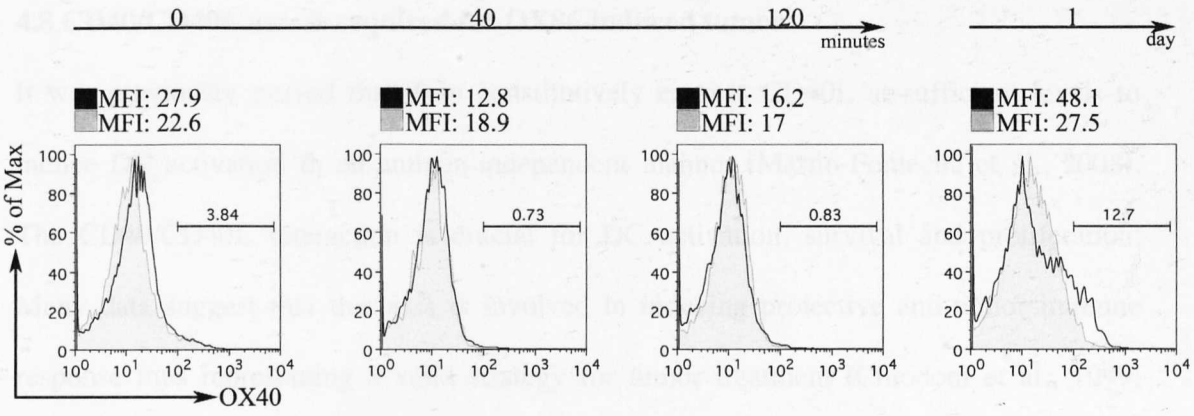


Figure 4.8: BM-DC-induced Tem do not up-regulate OX40. BALB/C mice were immunized with two consecutive injection of 10^6 BM-DC, pre-activated *in vitro* with 100 ng/ml LPS. One month later Tem were sorted form total splenocytes as $CD4^+CD44^{high}CD62L^{low}$ cells. OX40 expression on Tem was evaluated on sorted unstimulated cells or at different time points upon activation with αCD3 and αCD28. Tem modestly up-regulated OX40 (open histogram) 24 hours upon activation, while no expression was detected at earlier time points. Filled grey histogram: isotype control. MFI: mean fluorescence intensity.

4.8 CD40/CD40L axis is required for OX86-induced tumor

It was previously proved that Tem constitutively express CD40L at sufficient levels to induce DC activation in an antigen-independent manner (Martin-Fontecha et al., 2008). The CD40/CD40L interaction is crucial for DC activation, survival and proliferation. Many data suggest that this axis is involved in inducing protective anti-tumor immune response thus representing a valid strategy for tumor treatment (Chiodoni et al., 1999; Hanig and Lutz, 2008; Mackey et al., 1998; Murugaiyan et al., 2007). To investigate whether OX86-induced tumor rejection was dependent on the CD40/CD40L axis, wt and $CD40^{-/-}$ mice were subcutaneously inoculated with CT26 cells and treated intratumorally with a single injection of OX86 or isotype matched control Ab. While OX86 treatment induced tumor rejection or impaired tumor growth in CD40-sufficient mice, in CD40-deficient mice it was completely ineffective, (Fig 4.9 A). These data clearly demonstrate that OX40 triggering may act by reinforcing the CD40/CD40L pathway, which in turn may foster anti-tumor immunity.

The CD40/CD40L axis has a bidirectional effect: indeed, on one side CD40 provides signals required for DC licensing, on the other side CD40L improves T cell activation. Therefore, in $CD40^{-/-}$ mice, Tem receiving no stimulation via CD40/CD40L may express lower levels of OX40 (whose expression level closely correlates with T cell activation status), compromising their responsiveness to OX86 treatment. To exclude this hypothesis, OX40 expression was evaluated on Tem isolated from CT26 tumors growth in both wt and $CD40^{-/-}$ mice. However no difference in OX40 expression level was found between the two subsets of Tem (Fig 4.9 B, C). These data further demonstrate that the failure of OX86 treatment in the absence of CD40 was not due to a defective activation of Tem, but to an inadequate DC stimulation. In particular CD40 deficient DC could be impaired in two different phases of tumor rejection: i) in tumor microenvironment $CD40^{-/-}$ DC may not be

adequately licensed thus not being able to migrate from the tumor to the dLN, ii) $CD40^{-/-}$ DC could be able to migrate to the dLN but impaired in their ability to induce new T cell priming, because licensed but not fully competent for optimal T cell costimulation.

To discriminate which of these two mechanisms was defective in $CD40^{-/-}$ mice, an *in vivo* DC migration assay was performed. Tumors growing in wt and $CD40^{-/-}$ mice were treated with OX86 or the control Ab co-injected with green fluorescent microbeads, which could be up-taken by DC. After 24 hours, DC migration from tumor to dLN was checked. In this experimental setting, only DC that have up-taken the beads at the tumor site could be detected as fluorescent in the dLN. Although OX86 rescued DC migration from tumor to dLN in wt mice, the same treatment was ineffective in $CD40^{-/-}$ mice (Fig 4.9 D), a finding implying that in absence of the CD40/CD40L axis tumor-associated DC cannot be activated.

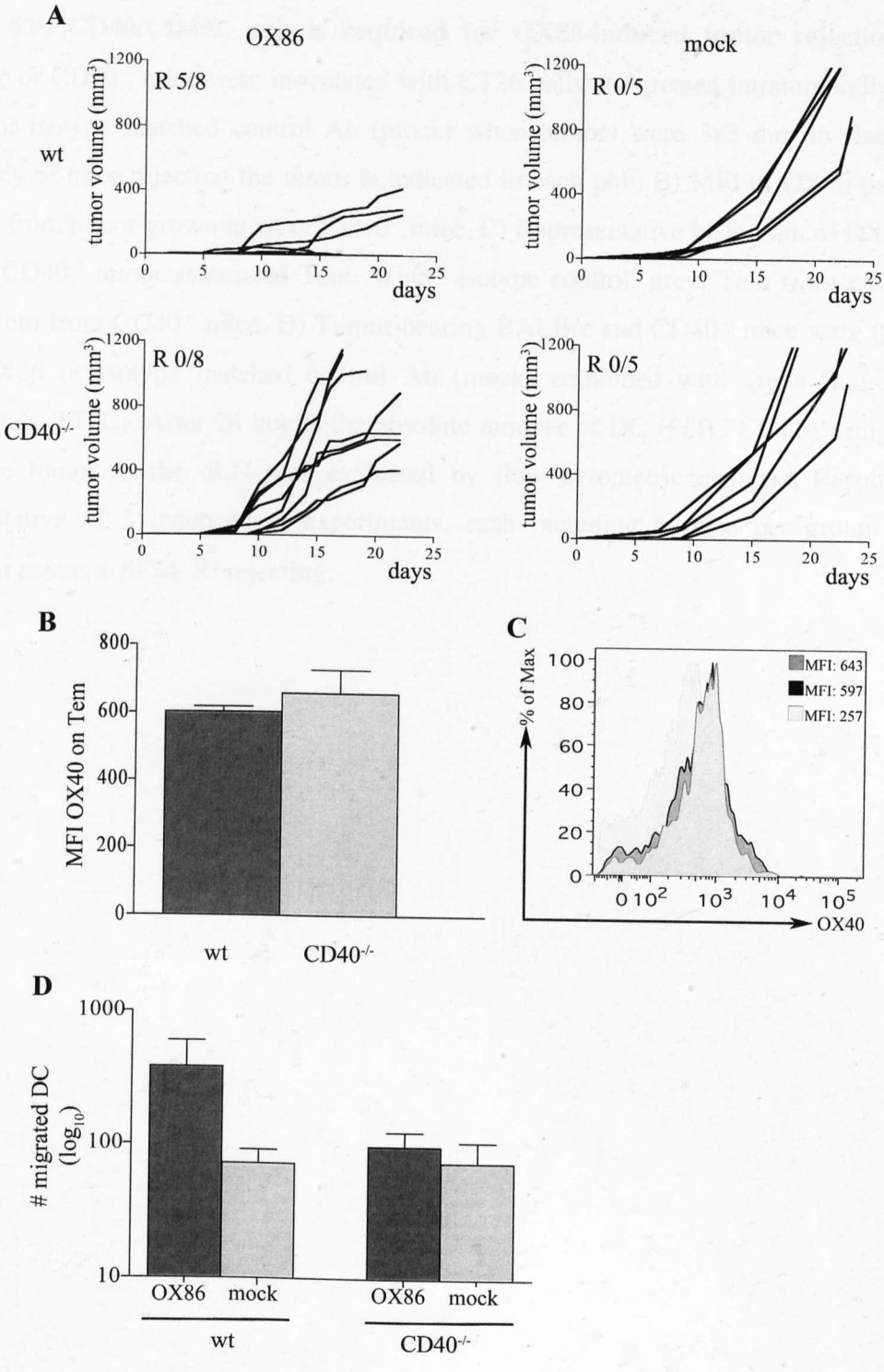

A
OX86
mock
wt
CD40-/-
R 5/8
R 0/5
R 0/8
R 0/5
tumor volume (mm3)
days
B
MFI OX40 on Tem
wt
CD40-/-
C
% of Max
MFI: 643
MFI: 597
MFI: 257
OX40
D
migrated DC (log10)
OX86
mock
wt
CD40-/-

Figure 4.9: CD40/CD40L axis is required for OX86-induced tumor rejection. A) BALB/c or $CD40^{-/-}$ mice were inoculated with CT26 cells and treated intratumorally with OX86 or isotype matched control Ab (mock) when tumors were 3x3 mm in size. The frequency of mice rejecting the tumor is indicated in each plot. B) MFI of OX40 on Tem isolated from tumor grown in wt or $CD40^{-/-}$ mice. C) Representative histogram of OX40 on wt and $CD40^{-/-}$ tumor-associated Tem. White: isotype control; grey: Tem from wt mice; black: Tem from $CD40^{-/-}$ mice. D) Tumor-bearing BALB/c and $CD40^{-/-}$ mice were treated with OX86 or isotype matched control Ab (mock) combined with green fluorescent microbeads (FITC). After 24 hours, the absolute number of DC ($FITC^{+}CD11c^{+}$) migrated from the tumor to the dLN was evaluated by flow cytometric analysis. Results are representative of 3 independent experiments, each including 6 mice per group. Bars represent means ± SEM. R: rejecting.

4.9 OX86 increases CD40L expression on Tem in tumor

We hypothesized that, in the immunosuppressive tumor microenvironment, Tem were inhibited in their ability to license DC via CD40L, and that OX40 triggering might provide the right signal for Tem to supply an effective CD40/CD40L mediated co-stimulation.

We found that the percentage of CT26 tumor-infiltrating Tem did not change 24 hours after the intratumoral injection of OX86 (Fig 4.10 A), so we checked whether the expression of the CD40L was modulated on Tem upon OX40 engagement. The detection of CD40L expression *ex vivo* may represent a crucial issue, as it is reported that CD40L rapidly disappears from cell surface, also following CD40 exposure (Castle et al., 1993; Lesley et al., 2006; Yellin et al., 1994). CD40L staining was done using two different methods: directly *ex vivo* or after TIL in vitro culture (Jaiswal and Croft, 1997; Jaiswal et al., 1996). In the first case TIL were incubated with CD40L mAb 1 hour on ice. In the second case TIL were incubated for 4 hours at 37°C with 4 γg/ml of anti-CD40L mAb. However no significant differences were found between the two methods in term of CD40L MFI (data not shown). All the data shown about CD40L expression were done directly *ex vivo*. We found that OX86 intratumoral injection induced a significant up-regulation of CD40L on the surface of Tem (Fig 4.10 B). Such CD40L up-modulation was specific for Tem, as no other T cell subsets, and especially $CD44^{low}CD46L^{low}$ recently activated T cells (Tact), responded similarly (Fig 4.10 C).

In the immunosuppressive tumor microenvironment T cells may become anergic and express the co-inhibitory molecule Pd1. Many data demonstrate that the blockade of this molecule reverts the inactive state of effector T cells and facilitates the development of an efficient anti-tumor immune response (Keir et al., 2008; Sakuishi et al., 2010; Woo et al., 2012). In this regard Pd1 expression was evaluated on T cells infiltrating CT26 tumor and dLN 24 hours after OX86 treatment. We found that OX40 triggering did not revert the

exhaustion status of TIL, as no difference in Pd1 expression was observed between OX86-treated and control TIL (Fig 4.10 D, E).

These data suggest that the lack of tumor rejection and DC migration upon OX86 treatment in $CD40^{-/-}$ is a consequence of insufficient DC reactivation in the tumor microenvironment, since OX86 does not recover T cells from their exhausted phenotype, but reinforces the CD40/CD40L axis.

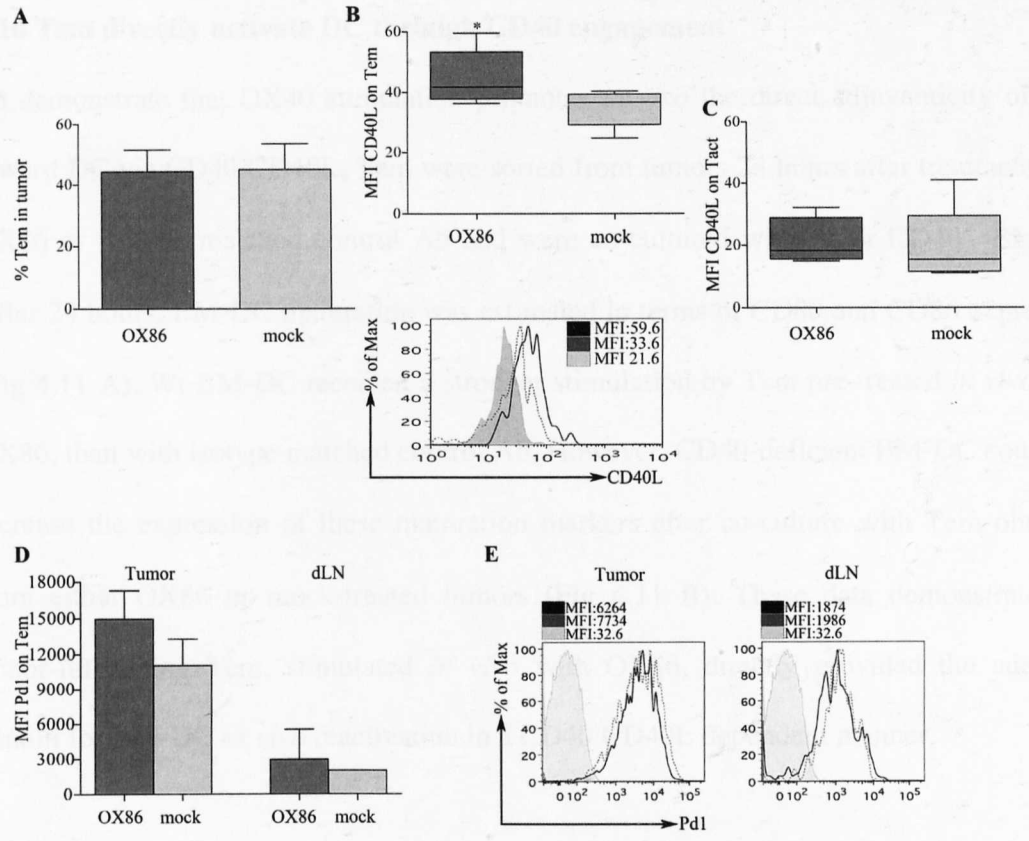


Figure 4.10: OX86 up-regulates CD40L specifically on tumor infiltrating Tem. A) BALB/c mice were inoculated with CT26 cells and treated intratumorally with OX86 or isotype matched control Ab (mock). 24 hours later the percentage of tumor infiltrating Tem was evaluated on the total $CD4^+$ T lymphocytes population enriched by ficoll gradient. B) Plot and representative histograms of CD40L MFI on tumor-infiltrating Tem 24 hours after OX86 or isotype matched Ab (mock) injection. Filled grey line: isotype control; dotted line: mock-treated Tem; black line: OX86-treated Tem. C) MFI of CD40L on tumor-infiltrating Tact 24 hours after OX86 or isotype matched Ab (mock) injection. D) Plot and E) representative histogram of MFI of Pd1 on Tem isolated from CT26 tumor mass and dLN treated with OX86 or isotype matched control Ab (mock). Filled grey line: isotype control; dotted line: Tem from mock-treated mice; black line: Tem from OX86-treated mice. Data are representative of three independent experiments each with 6 mice. Bars represent means ± SEM. *$p<0.05$, two-tailed Student's t-test. MFI= mean fluorescence intensity, dLN: draining lymph node.

4.10 Tem directly activate DC through CD40 engagement

To demonstrate that OX40 stimulation promotes *in vivo* the direct adjuvanticity of Tem toward DC via CD40/CD40L, Tem were sorted from tumors 24 hours after treatment with OX86 or isotype matched control Ab and were co-cultured with wt or $CD40^{-/-}$ BM-DC. After 24 hours, BM-DC maturation was estimated in terms of CD80 and CD86 expression (Fig 4.11 A). Wt BM-DC received a stronger stimulation by Tem pre-treated *in vivo* with OX86, than with isotype matched control Ab. However, CD40-deficient BM-DC could not increase the expression of these maturation markers after co-culture with Tem obtained from either OX86 or mock-treated tumors (Fig 4.11 B). These data demonstrate that tumor-infiltrating Tem, stimulated *in vivo* with OX86, directly provided the adequate stimuli for BM-DC *ex vivo* reactivation in a CD40/CD40L dependent manner.

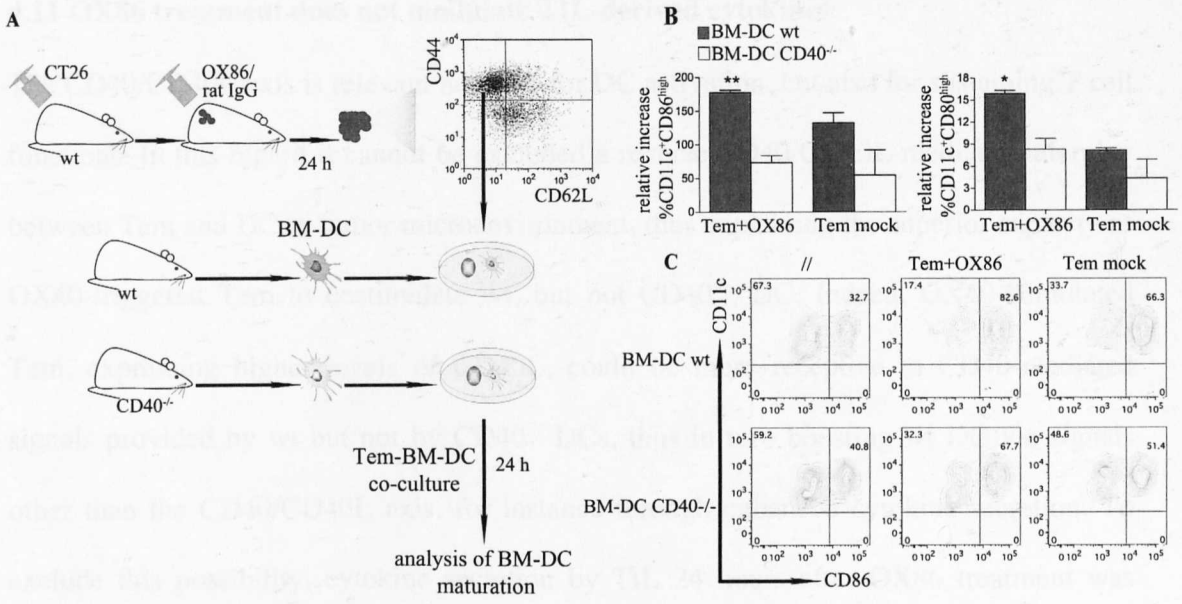


Figure 4.11: Tem activate BM-DC more efficiently after OX40 triggering in a CD40/CD40L dependent manner. A) Experimental scheme. BALB/c mice were inoculated with CT26 cells, treated with OX86 or isotype matched control Ab (mock). After 24 hours, Tem were sorted from TIL and co-cultured for 24 hours with wt or $CD40^{-/-}$ BM-DC at 1:1 ratio and BM-DC maturation was assessed in terms of CD80 and CD86 up-regulation. B) BM-DC activation status after co-culture with *in vivo*-treated tumor-infiltrating Tem. BM-DC maturation was evaluated as the percentage of the relative increase (compared to the corresponding not stimulated BM-DC) of the percentage of $CD11c^{+}CD80^{+}$ and $CD11c^{+}CD86^{+}$ cells. C) Representative plots of CD86 expression by wt or $CD40^{-/-}$ BM-DC in each culture condition. Results are from one of three independent experiments, each including 7 mice per group. BM-DC: bone marrow derived dendritic cells. Bars represent means ± SEM. $*p<0.05$ $**p<0.01$ by two-tailed Student's t test.

4.11 OX86 treatment does not modulate TIL-derived cytokines

The CD40/CD40L axis is relevant not only for DC activation, but also for sustaining T cell functions. In this regard it cannot be excluded a reverse CD40/CD40L-mediated interplay between Tem and DC in tumor microenvironment, thus explaining the superior capacity of OX40-triggered Tem to costimulate wt, but not $CD40^{-/-}$, DC. Indeed, OX40-stimulated Tem, expressing higher levels of CD40L, could be more receptive to CD40-mediated signals provided by wt but not by $CD40^{-/-}$ DCs, thus in turn boosting wt DC via signals other than the CD40/CD40L axis, for instance through enhanced cytokine secretion. To exclude this possibility, cytokine secretion by TIL 24 hours after OX86 treatment was evaluated by ICS upon *in vitro* restimulation with PMA, IONO and Monensin. No increase in the production of IFNγ, TNFα, IL-17 or IL-6 by TIL *ex vivo* was found upon *in vivo* OX86 injection (Fig 4.12 A-H). These data demonstrate that the increased capacity of OX86-stimulated Tem to activate DC is tightly dependent on their up-regulation of CD40L and does not require other activator stimuli provided by DC via CD40 or higher concentration of inflammatory cytokines.

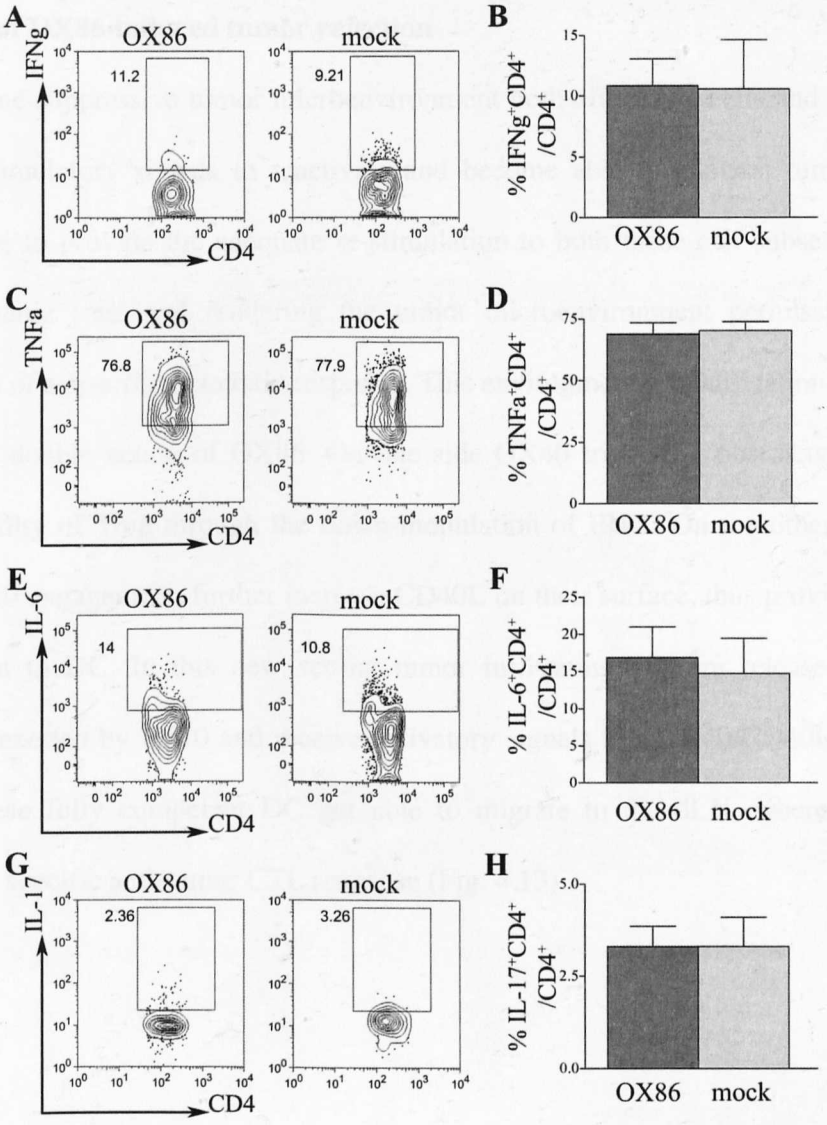


Figure 4.12: OX86 does not modulate cytokine secretion by TIL. BALB/c mice were subcutaneously inoculated with CT26 tumor cells and subsequently treated with OX86 or isotype matched control Ab (mock). TIL were purified from tumor mass by ficoll gradient and restimulated in vitro with PMA, IONO and Monensin. Representative plots and percentage of TIL secreting IFNγ (A-B), TNFα (C-D), IL-6 (E-F) and IL-17 (G-H) show that OX86 does not modulate the cytokine production by TIL upon OX40 triggering. Data are representative of two independent experiments, which include 5 mice pour group. Bars represent means ± SEM. TIL: tumor-infiltrating lymphocytes.

4.12 Model of OX86-induced tumor rejection

In the immune-suppressive tumor microenvironment both effector T cells and DC require additional stimulatory signals to reactivate and become able to contrast tumor growth. OX86 is able to provide the adequate re-stimulation to both these cell subsets, breaking their tolerogenic state and rendering the tumor microenvironment permissive for the development of a specific cytotoxic response. This environmental modification is obtained thank to the double action of OX86. On one side OX40 triggering obstructs the IL-10-secretory ability of Treg through the down-modulation of IRF1. On the other side Tem, sensing OX40 engagement, further increase CD40L on their surface, thus providing strong costimulation to DC. In this new setting tumor infiltrating DC are released from the suppression exerted by IL-10 and receive activatory signals in a CD40/CD40L-dependent manner. These fully competent DC get able to migrate to the dLN, where efficiently activate new specific anti-tumor CTL response (Fig. 4.13).

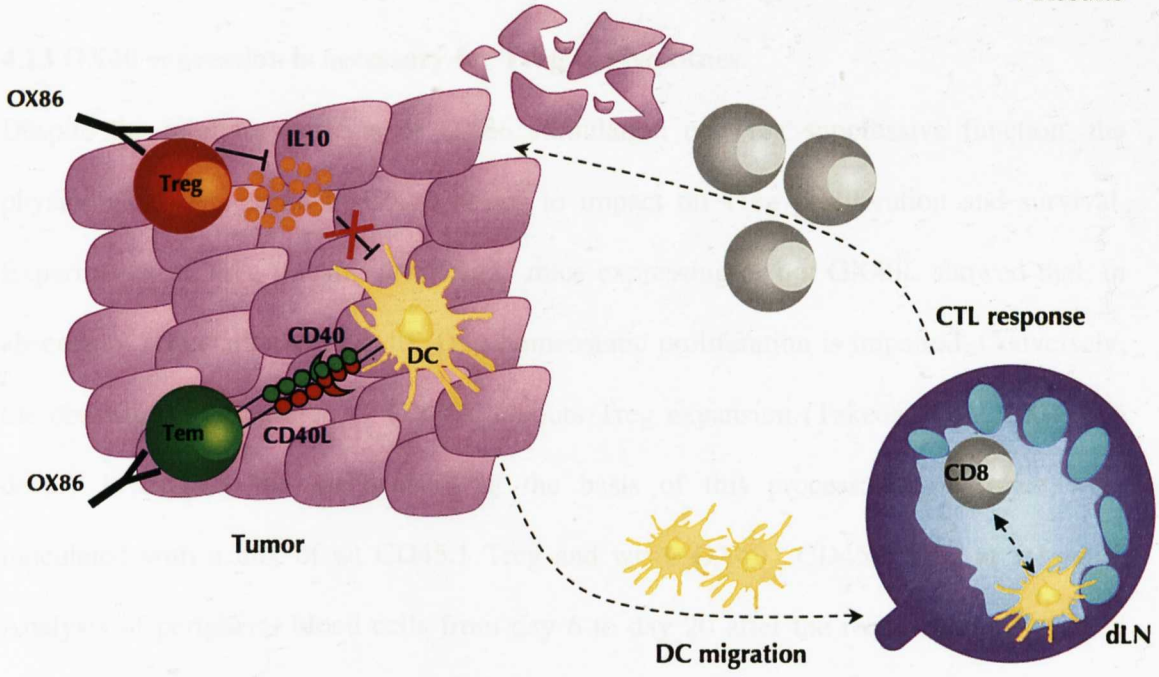


Figure 4.13: OX86-induced tumor rejection. OX86 intra-tumoral injection impairs IL-10-production by Treg through the down-modulation of IRF1. On the other side OX40 triggering further increases CD40L expression on Tem. In this favorable environment tumor infiltrating DC receive low inhibition via IL-10 and higher stimulation via CD40/CD40L, thus becoming able to migrate from the tumor to the dLN and activate new specific anti-tumor CTL response.

4.13 OX40 expression is necessary for Treg in vivo fitness

Despite the inhibitory effects of OX86 stimulation on Treg suppressive function, the physiological expression of OX40 seems to impact on Treg proliferation and survival. Experiments of Treg transfer into $Rag2^{-/-}$ mice expressing or not OX40L showed that, in absence of stimulation via OX40, Treg homeostatic proliferation is impaired. Conversely, the constitutive expression of OX40L favours Treg expansion (Takeda et al., 2004). To deeply investigate the mechanisms at the basis of this process, $Rag1^{-/-}$ mice were inoculated with a mix of wt CD45.1 Treg and wt or $OX40^{-/-}$ CD45.2 Treg at 1:1 ratio. Analysis of peripheral blood cells from day 6 to day 20 after the reconstitution revealed that, in the absence of OX40, Treg had a disadvantage in proliferation compared to wt Treg. On the contrary the homeostatic proliferation of wt and $OX40^{-/-}$ $CD45.2^+CD4^+Foxp3^-$ effector cells, expanded *in vivo* from the respective $CD45.2^+CD4^+Foxp3^+$ cell counterpart after adoptive transfer into $Rag1^{-/-}$ mice, did not display any difference. These data suggest that OX40 deficiency specifically impairs Treg proliferation, without affecting the proliferative potential of effector cells (Fig. 4.14).

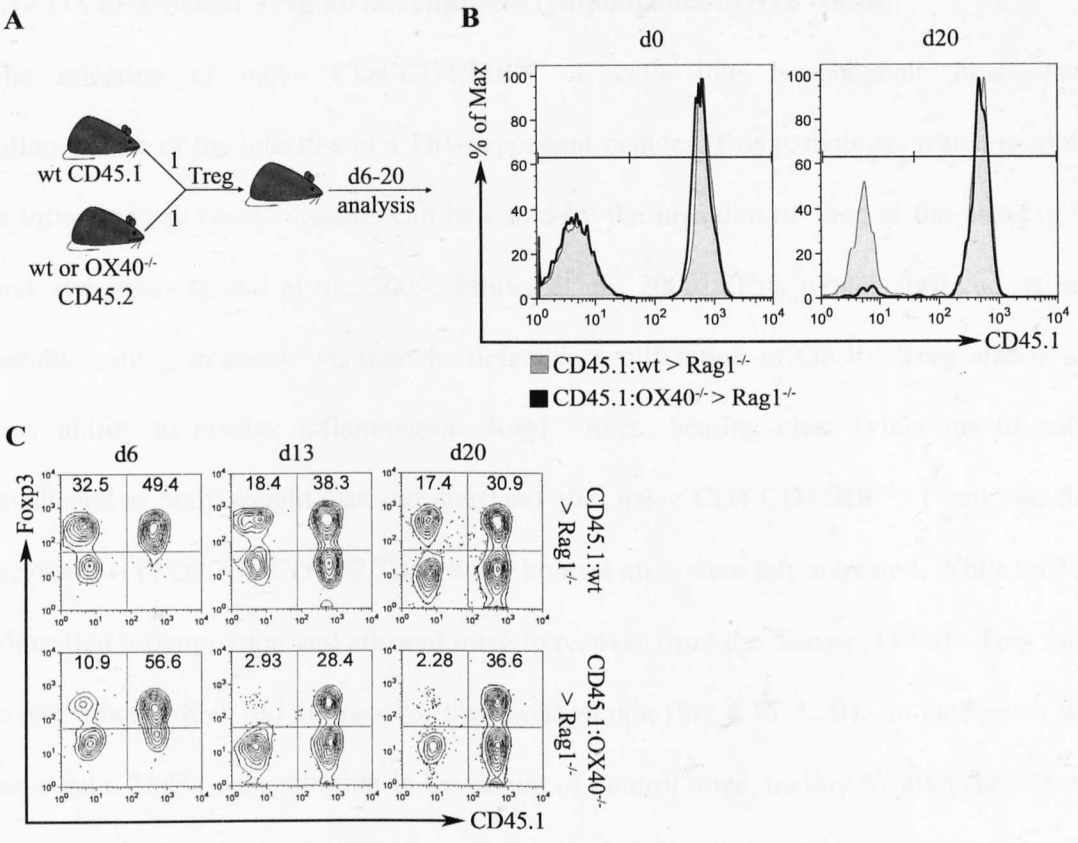


Figure 4.14: OX40-expressing Treg have higher fitness compared to OX40$^{-/-}$ Treg in lymphopenic condition. A) Experimental scheme. Rag1$^{-/-}$ mice were i.v injected with a 1:1 mix of wt CD45.1 Treg and wt or OX40$^{-/-}$ CD45.2 Treg. Treg expansion was evaluated by FACS analysis of peripheral blood at the indicated time points (day 6, 13 and 21 after transfer). B) Representative histograms of CD45.1 and wt or OX40$^{-/-}$ CD45.2 Treg frequency before and 20 days after the transfer into recipient mice. C) Representative plots of circulating CD45.1^{+} Foxp3^{+} and wt or OX40$^{-/-}$ CD45.2^{+} Foxp3^{+} cells 6, 13 and 21 days after transfer into Rag1$^{-/-}$ mice. Data are from one of two independent experiments, each including four mice per group. d: day.

4.14 OX40 deficient Treg do not suppress lymphopenia-driven colitis

The injection of naïve $CD4^+CD45RB^{high}$ T cells into lymphopenic host causes inflammation of the intestine in a Th1-dependent manner. This pathology, which is similar to inflammatory bowel disease, can be cured by the injection of Treg at the onset of the first symptoms (Izcue et al., 2009; Mottet et al., 2003). This model appeared to be a suitable setting to check whether the defective proliferation of $OX40^{-/-}$ Treg affects also their ability to resolve inflammation. $Rag1^{-/-}$ mice, bearing clear symptoms of colitis (evaluated as body weight loss and diarrhea) after naïve $CD4^+CD45RB^{high}$ T cell injection, received wt or $OX40^{-/-}$ CD45.2 Treg, while control mice were left untreated. While wt Treg controlled inflammation and allowed mice to recover from the disease, $OX40^{-/-}$ Treg failed to cure mice, which did not recover the initial weight (Fig 4.15 A, B). In agreement with these data, TNFα concentration in the serum of control mice, on day 37 after the onset of the disease, was significantly higher compared to that of mice treated with wt, but not $OX40^{-/-}$, Treg (Fig 4.15 C). Histological scoring on the distal portion of the colon on day 42 mirrored TNFα concentration among the three groups of mice. Untreated mice displayed complete disruption of glands, intense inflammatory infiltration and significant reduction in the number of goblet cells. Mice cured with wt Treg had limited leukocyte infiltration, moderate crypt hyperplasia and almost normal number of goblet cells. Mice treated with $OX40^{-/-}$ Treg still had consistent leukocytes infiltration, mainly in the lamina propria, moderate hyperplasia of the crypts and reduced number of goblet cells (Fig 4.15 D, E).

This experiment confirms that OX40 deficiency impairs Treg ability to cure inflammation, possibly as a consequence of their reduced proliferative potential.

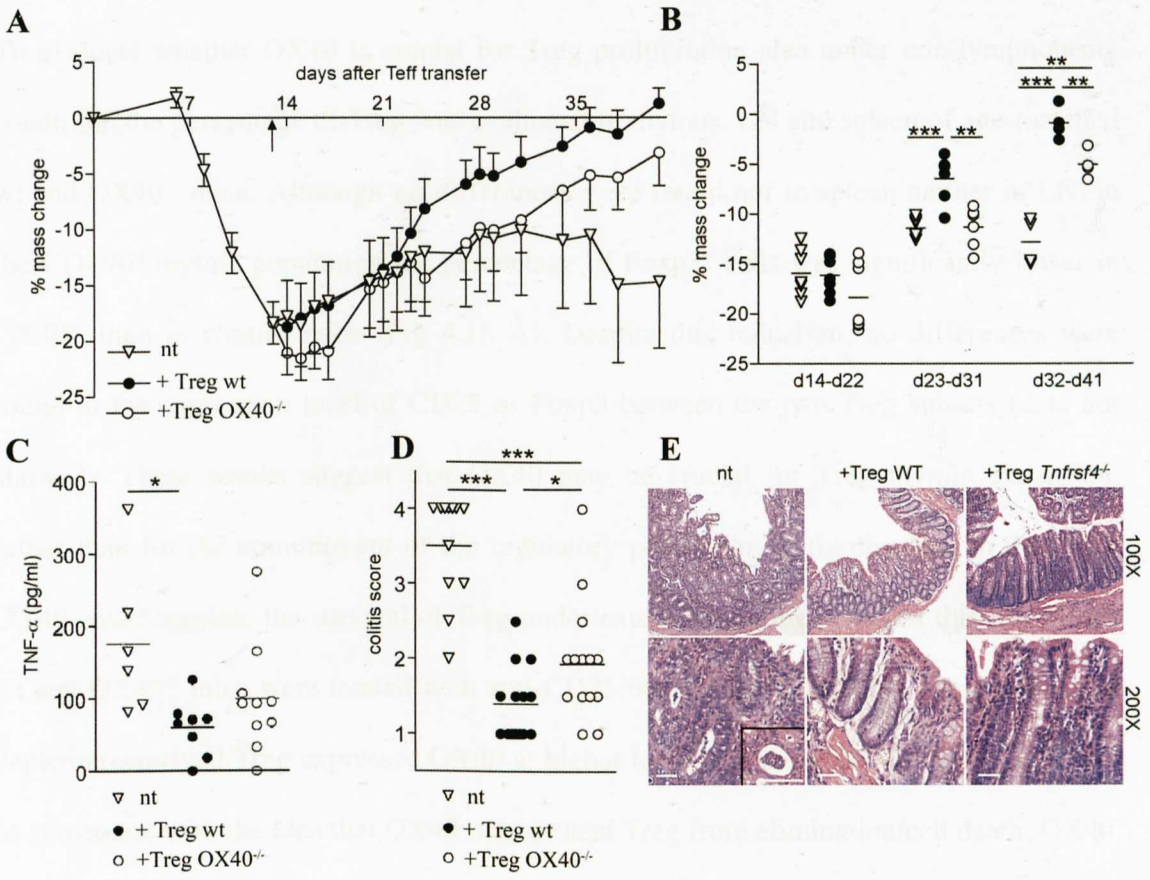


Figure 4.15: OX40$^{-/-}$ Treg show impaired ability to cure colitis. Rag1$^{-/-}$ mice were i.p injected with sorted CD45.1^{+}CD45RB^{high} lymphocytes at day 0. At the onset of the first symptoms (day 13, arrow) mice were left untreated (nt) or received wt or OX40$^{-/-}$ CD45.2 Treg. A) Percentage of body weight change in the three groups of mice along all the time of disease progression (mean ± SD). B) Percentage of body weight change in three time intervals, each symbol corresponds to one mouse. C) Serum concentration of TNFα on day 37 of colitis (mean ± SD). D) Histological grading of colitis on day 42 and E) H&E staining on distal portion of colon. Scale bars (white): 50mm. Original magnification: upper panels 100X, lower panels 200X, inset 400X. Data are representative of two independent experiments with 4-5 mice per group. * $p<0.05$, ** $p<0.001$, *** $p<0.005$, two-tailed Student's t test. nt: not treated

4.15 OX40 deficiency impairs Treg proliferation also in physiological condition

To evaluate whether OX40 is crucial for Treg proliferation also under non-lymphopenic condition, the percentage of Treg was evaluated in thymus, LN and spleen of age-matched wt and $OX40^{-/-}$ mice. Although no differences were found nor in spleen neither in LN, in the $CD4^{+}SP$ thymic population the percentage of $Foxp3^{+}$ cells was significantly lower in $OX40^{-/-}$ than in control mice (Fig 4.16 A). Despite this reduction, no differences were found in the expression level of CD25 or Foxp3 between the two Treg subsets (data not showed). These results suggest that OX40 may be crucial for Treg thymic expansion, rather than for the commitment of the regulatory population in the thymus. In this view OX40 could regulate the survival of Treg under external pressures. To test this hypothesis wt and $OX40^{-/-}$ mice were treated with anti-CD25 mAb (PC61). In PC61-treated wt mice, depletion-survived Treg expressed OX40 at higher level than control Treg (Fig 4.16 B, C). In agreement with the idea that OX40 may protect Treg from elimination/cell death, $OX40^{-/-}$ Treg were more susceptible to PC61-driven depletion than wt Treg (Fig 4.16 D, E). The inefficient proliferation of $OX40^{-/-}$ Treg was checked also in thymectomized mice. Three weeks following the surgical thymectomy, the frequency of Treg was significantly higher in wt mice compared to $OX40^{-/-}$ mice (Fig 4.16 F), since in absence of OX40 Treg showed reduced proliferation rate as measured by BrdU incorporation (Fig 4.16 G) and higher apoptosis (Fig 4.16 H). All together these data suggest that OX40 plays a fundamental role in regulating Treg fitness, in both lymphopenic and non-lymphopenic hosts, maybe by modulating the sensitivity to survival signals, for instance to IL-2, which is the most relevant cytokine for Treg survival.

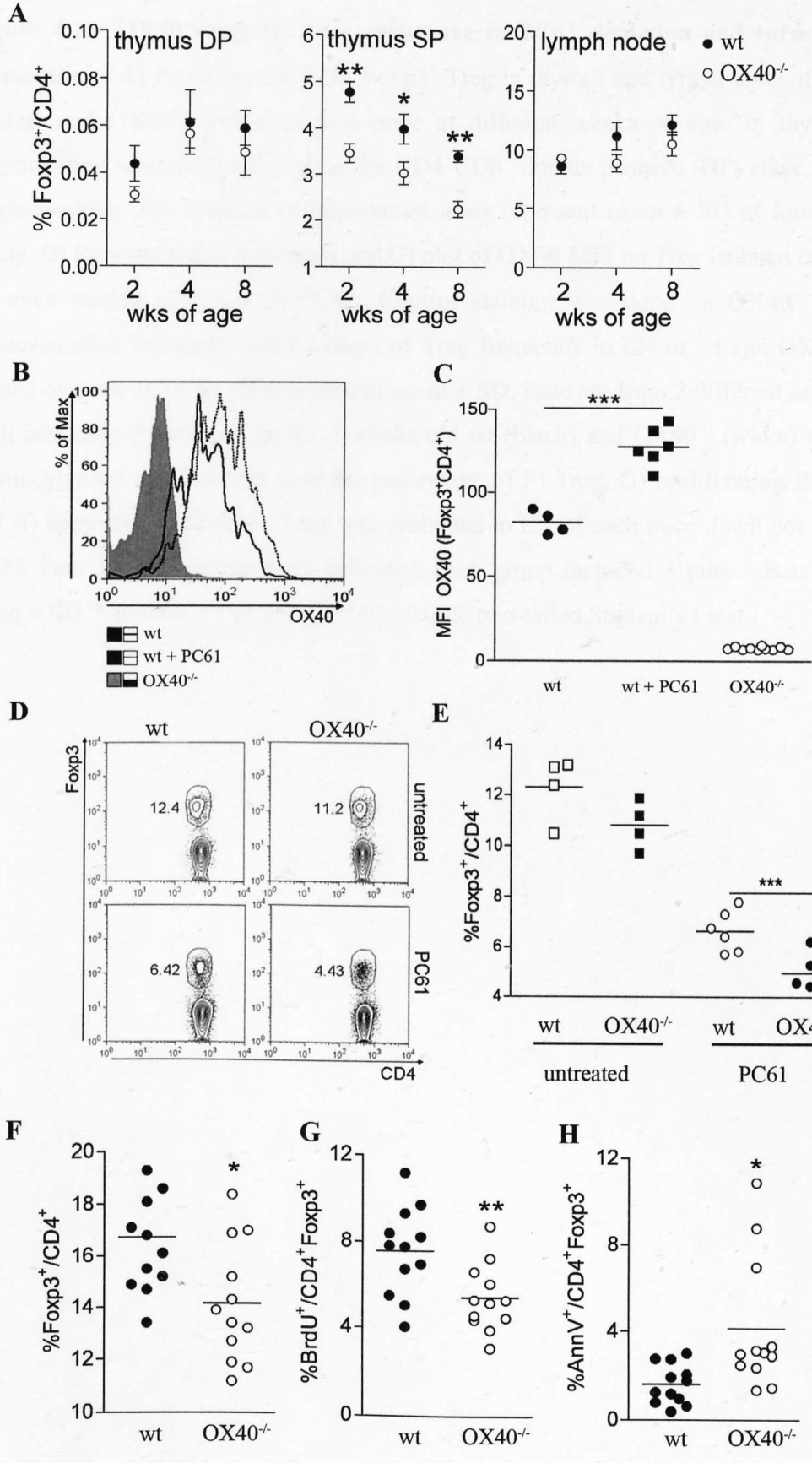

A
thymus DP
thymus SP
lymph node
wt
OX40-/-
% Foxp3+/CD4+
wks of age
B
% of Max
OX40
wt
wt + PC61
OX40-/-
C
MFI OX40 (Foxp3+CD4+)
***
wt
wt + PC61
OX40-/-
D
wt
OX40-/-
Foxp3
CD4
untreated
PC61
12.4
11.2
6.42
4.43
E
%Foxp3+/CD4+
***
wt
OX40-/-
untreated
PC61
F
%Foxp3+/CD4+
G
%BrdU+/CD4+Foxp3+
H
%AnnV+/CD4+Foxp3+

Figure 4.16: OX40 supports Treg resistance to PC61 depletion and turnover after thymectomy. A) Percentage of $CD4^+Foxp3^+$ Treg in thymus and lymph node of wt (black circles) and $OX40^{-/-}$ (white circles) mice at different weeks of age. In thymus Treg accumulation was evaluated both at the $CD4^+CD8^+$ double positive (DP) stage and $CD4^+$ single positive (SP) stage of differentiation. Bars represent mean ± SD of four mice per group. B) Representative histogram and C) plot of OX40 MFI on Treg isolated from LN of wt mice treated or not with PC61. Control staining was done on $OX40^{-/-}$ Treg. D) Representative histogram and E) graph of Treg frequency in LN of wt and $OX40^{-/-}$ mice treated or not with PC61. Bars represent mean ± SD. Date are from 2 different experiments each including 5 mice per group. 5-weeks old wt (black) and $OX40^{-/-}$ (white) mice were thymectomized and 3 weeks later the percentage of F) Treg, G) proliferating $BrdU^+$ Treg and H) apoptotic Annexin V^+ Treg was evaluated in LN of each mice. Each dot represents a LN. Four LN per mouse were collected, each group included 3 mice. Bars represent mean ± SD. * $p<0.05$, ** $p<0.001$, *** $p<0.005$, two-tailed Student's t test..

4.16 OX40 regulates Treg sensitivity to IL-2

To check the possibility that OX40 mediates Treg-sensitivity to IL-2, wt and $OX40^{-/-}$ Treg were *in vitro* stimulated with increasing doses of recombinant (r) IL-2. Responsiveness to IL-2 was evaluated in term of Stat5 phosphorylation (pStat5), estimated by flow cytometric analysis, at different time points (1, 5, 10 minutes) upon stimulation with r-IL-2. Absence of OX40 significantly impairs the ability of Treg to sense IL-2, mainly in the later phases of the response, as the defect was more evident at 5-10 minutes than after 1 minute of stimulation (Fig 4.17 A, B). It has been demonstrated that SOCS1 inhibits Stat5 phosphorylation (Sporri et al., 2001), and that the microRNA155 (miR155), induced by Foxp3, has SOCS1 as target (So et al., 2011a). Thus, the reduced fitness of $OX40^{-/-}$ Treg could be due to a deregulation of this mechanism. To verify this hypothesis, SOCS1 and miR155 expression were evaluated by western blot and qRT_PCR, respectively, in wt and $OX40^{-/-}$ Treg (Fig 4.17 C, D). In $OX40^{-/-}$ Treg higher levels of SOCS1 and lower expression of miR155 were found. These data confirm that the absence of OX40 has a negative impact on Treg, which display an imbalance between SOCS1 and miR155 and become less sensitive to IL-2 signal, as demonstrated by the reduction in Stat5 phosphorylation.

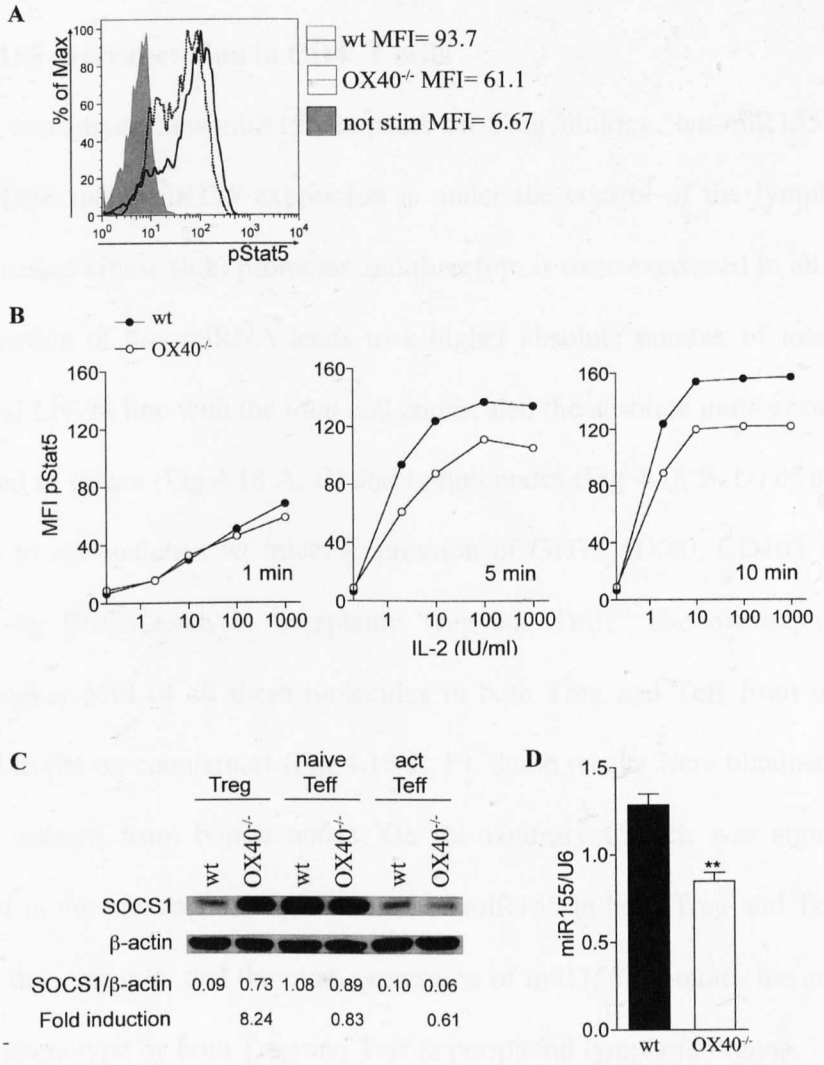


Figure 4.17: OX40 deficiency attenuates Treg responsiveness to IL-2. A) Representative histogram and B) plot of pStat5 MFI in wt and OX40$^{-/-}$ purified Treg untreated or stimulated with scaled doses of r-IL-2 at different time points. C) Western blot for SOCS1 in Treg, naïve and activated effector T cells (Teff) purified from wt or OX40$^{-/-}$ mice. Normalized SOCS1 expression and fold induction are reported for each sample. D) miR155 expression in wt and OX40$^{-/-}$ Treg was evaluated by quantitative real-time PCR and normalized to U6. Data are representative of two different experiments each including 5-10 mice per group. **p<0.01, two-tailed Student's t test.

4.17 miR155 overexpression in CD4$^+$ T cells

To better investigate how miR155 impacts on Treg biology, lck-miR155 tg mice were used. In these mice miR155 expression is under the control of the lymphocyte-specific protein tyrosine kinase (lck) promoter and therefore is over-expressed in all T cell subsets. Overexpression of this miRNA leads to a higher absolute number of total cells in both spleens and LN. In line with the total cell count, also the absolute number of Teff and Treg is increased in spleen (Fig 4.18 A, C) and lymph nodes (Fig 4.18 B, D) of miR155 tg mice compared to age-matched wt mice. Expression of GITR, OX40, CD103 and CD69 was evaluated by FACS analysis on splenic Treg and Teff. The overexpression miR155 induced higher MFI of all these molecules in both Treg and Teff from transgenic mice compared to the wt counterpart (Fig 4.18 E, F). Same results were obtained for both Treg and Teff isolated from lymph nodes. On the contrary CD62L was significantly down modulated in the presence of high amount of miR155 in both Treg and Teff (Fig 4.18 E, F). These data suggests that the over-expression of miR155 promotes the acquisition of an activated phenotype by both Treg and Teff in peripheral lymphoid organs.

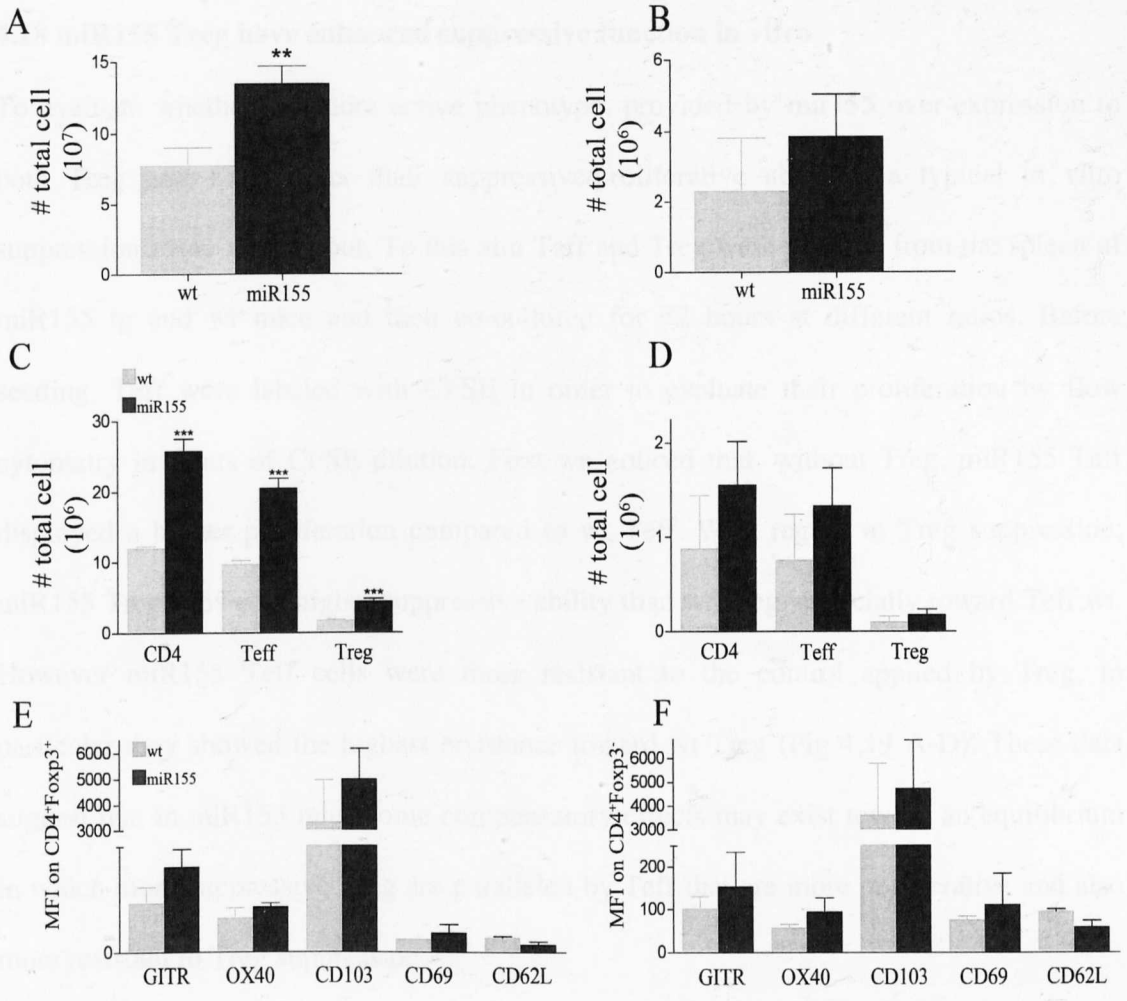


Figure 4.18: miR155 over-expression leads to accumulation of highly activated Treg and Teff in peripheral lymphoid tissues. Total absolute cell number in spleen (A) and LN (B) of age-matched miR155 tg and wt mice. Absolute number of $CD4^+$, Teff and Treg in spleen (C) and LN (D) of age-matched miR155 tg and wt mice. E-F) MFI of GITR, OX40, CD103, CD69, CD62L on splenic Treg (E) and Teff (F) from miR155 tg (black) and wt (grey) mice. Bars represent means ± SD, data are representative of 3 independent experiments, each including 3 mice per group. $^{**}p<0.01$, $^{***}p<0.005$ two-tailed Student's t test.

4.18 miR155 Treg have enhanced suppressive function in vitro

To evaluate whether the more active phenotype, provided by mir155 over-expression to both Treg and Teff, alters their suppressive/proliferative abilities, a typical in vitro suppression assay was set out. To this aim Teff and Treg were purified from the spleen of miR155 tg and wt mice and then co-cultured for 72 hours at different ratios. Before seeding, Teff were labeled with CFSE in order to evaluate their proliferation by flow cytometry in terms of CFSE dilution. First we noticed that, without Treg, miR155 Teff displayed a higher proliferation compared to wt Teff. With regard to Treg suppression, miR155 Treg showed a higher suppressive ability than wt Treg, especially toward Teff wt. However miR155 Teff cells were more resistant to the control applied by Treg, in particular they showed the highest resistance toward wt Treg (Fig 4.19 A-D). These data suggest that in miR155 mice some compensatory effects may exist toward an equilibrium in which more suppressive Treg are paralleled by Teff that are more proliferative and also more resistant to Treg suppression.

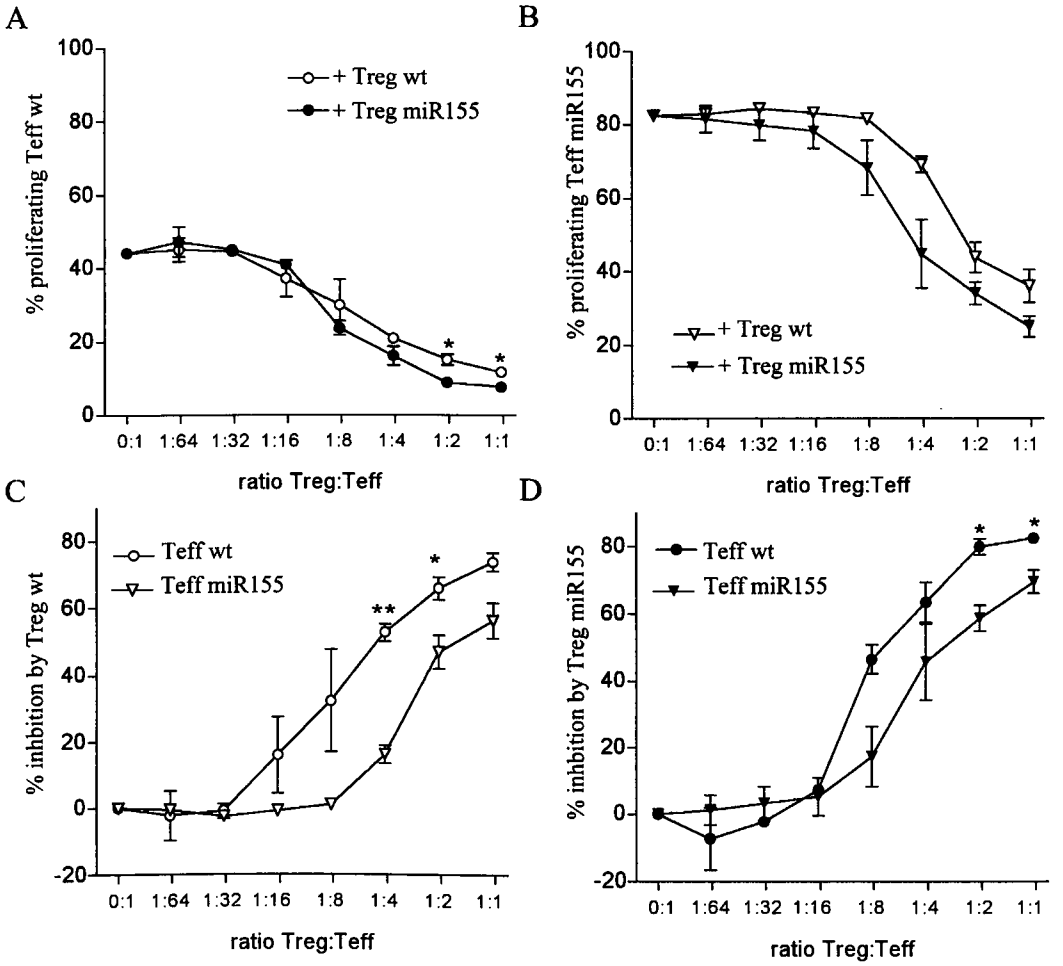


Figure 4.19: miR155 Treg displayed higher suppression, compensated by highly resistant Teff. Treg and Teff were purified from the spleen of miR155 tg or wt mice. Teff were labeled with CFSE and seeded with Treg at different ratios (Treg:Teff 0:1, 1:64, 1:32, 1:16, 1:8, 1:4, 1:2, 1:1) for 72 hours in the presence of accessory cells (AC, irradiated splenocytes) and soluble αCD3. Teff proliferation was evaluated as CFSE dilution by flow cytometry. A) Percentage of proliferating wt Teff co-cultured with miR155 or wt Treg. B) Percentage of proliferating miR155 Teff co-cultured with miR155 or wt Treg. C) Percentage of inhibition exerted by wt Treg toward miR155 or wt Teff. D) Percentage of inhibition exerted by miR155 Treg toward miR155 or wt Teff. Data are representative of 3 different experiments. Bars represent mean ± SD. *p<0.05, **p<0.01, two-tailed Student's t test.

4.19 Tumor growth in miR155 tg mice

To evaluate which of the above-described mechanisms prevails *in vivo,* i.e. the increased suppressive ability of Treg or the stronger resistance to suppression of Teff, miR155 tg and wt mice were subcutaneously inoculated with MCA38 or B16/F10 tumor cells. Although in miR155 mice the rate of tumor growth was not significantly higher compared to that of control mice, there was a faster progression, suggesting that *in vivo* the inhibitory function of miR155 Treg exceeded the ability of Teff to resist to the suppression. Analysis of TIL did not show any relevant differences in term of Teff/Treg ratio between miR155 tg and wt mice. Although additional experiments are required to better understand how miR155 influences Treg and Teff behavior, these data suggest that the over-expression of miR155 mainly modulates T cell functions, for instance in terms of cytokine secretion, rather that their proliferation/survival.

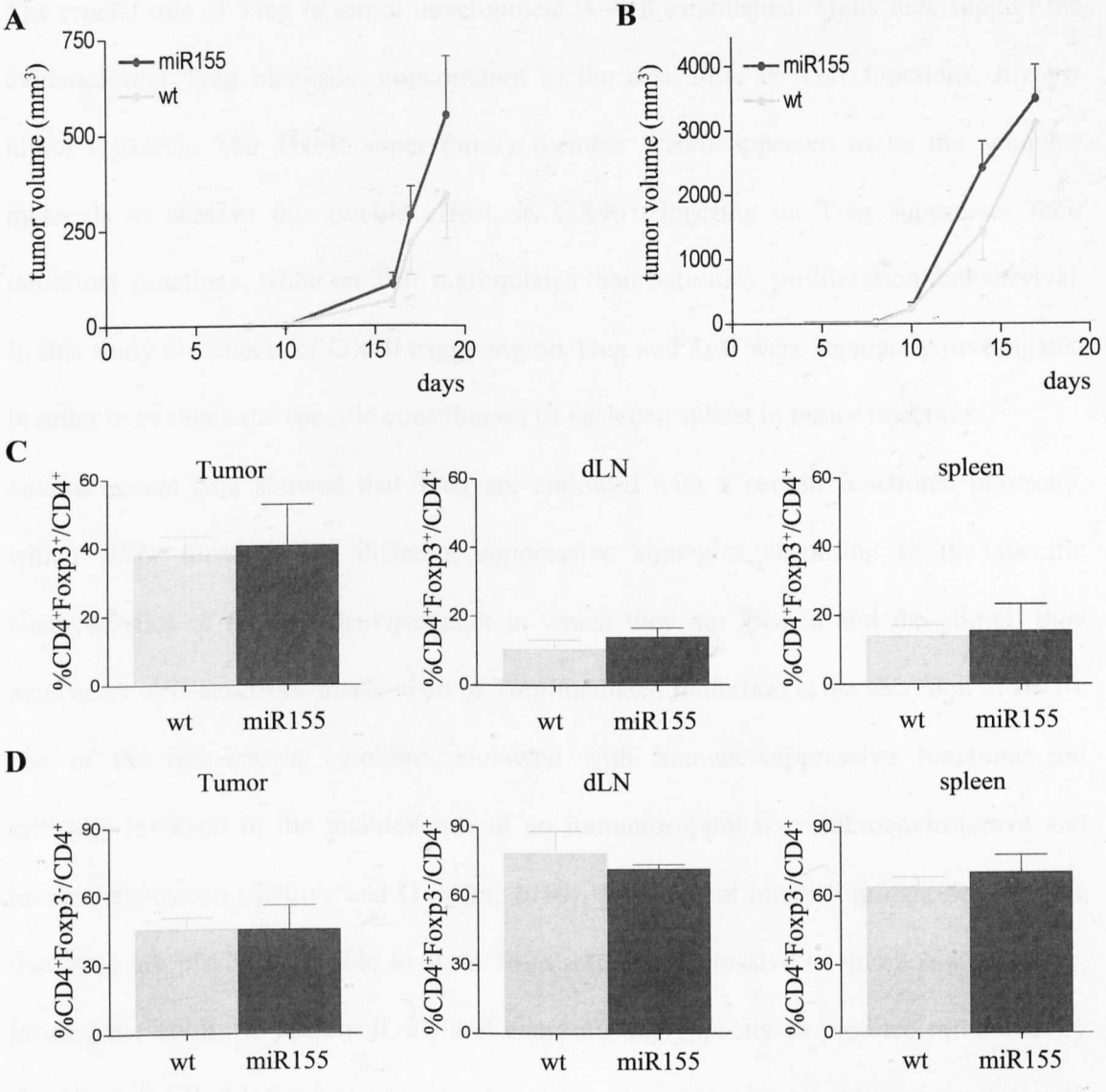


Figure 4.20: tumor growth in mir155 tg mice. miR155 tg and wt mice were subcutaneously inoculated with A) MCA38 colon cancer or B) B16/F10 melanoma tumor cells. Tumor growth was monitored every two days. C) Frequency of Treg ($CD4^+Foxp3^+$) and D) Teff ($CD4^+Foxp3^-$) in tumor, dLN and spleen of MCA38-bearing mice. Bars represent mean ± SD. Data are representative of 2 different experiments each including 6 mice per group.

5 Discussion

The crucial role of Treg in tumor development is well established. Many data support the evidence that Treg blockade, concomitant to the activation of Teff functions, favours tumor rejection. The TNFR super family member OX40 appeared to be the adequate molecule to achieve this double effect, as OX40 triggering on Treg suppresses their inhibitory functions, while on Teff it stimulates their activities, proliferation and survival. In this study the effects of OX40 triggering on Treg and Teff were separately investigated in order to evaluate the specific contribution of each cell subset in tumor rejection.

Several recent data showed that Treg are endowed with a certain functional plasticity, which allow them to use different suppressive strategies according to the specific characteristics of the microenvironment in which they are located and the stimuli they receive. A well-described mechanism of Treg-mediated inhibition is the secretion of IL-10, one of the best-known cytokines endowed with immune-suppressive functions and critically involved in the maintenance of an immunosuppressive microenvironment and tumor progression (Saraiva and O'Garra, 2010). Very recent interesting data demonstrate that Treg are plastic cells able to skew from immune-depressive to immune-stimulatory, loosing the ability to secrete IL-10 and acquiring the capacity to produce inflammatory cytokines like IL-17. For instance, in colon polyposis, under chronic inflammatory stimuli, Treg become pro-inflammatory and pro-tumorigenic, becoming able to produce IL-17 (Gounaris et al., 2009). Focusing on immune-suppressive molecules, in mouse transplantable tumor models it has been proven that IL-10 has a pro-tumoral role, preventing DC to become fully competent for orchestrating T cell responses (Dercamp et al., 2005). Although *Il10* gene was described as characteristic of Treg gene signature (Fontenot et al., 2005b), IL-10 secretion was well assessed only in intestine and lung of naïve mice (Maynard et al., 2007; Rubtsov et al., 2008; Uhlig et al., 2006). The present study demonstrates, for the first time, that Treg spontaneously secrete high amounts of IL-

10 in the tumor microenvironment. Notably Treg isolated from tumor-draining LN or spleen of tumor-bearing mice do not display such property, suggesting that Treg are able to produce IL-10 transiently only upon adequate stimuli provided in peculiar milieus, like the tumor microenvironment.

In a murine tumor model, it has been showed that $CD4^+CD25^+$ Treg and IL-10 have distinct, and partially overlapping, roles in keeping $CD8^+$ T cells unable to kill tumor cells and in blocking DC maturation (Dercamp et al., 2005). Although Treg depletion by means of Foxp3-targeting, rather than the CD25-directed Treg depletion, could provide more precise results on the specific contribution of Treg and IL-10 in tumor immunity, this result indicates that Treg are not the only source of IL-10 in tumor (Dercamp et al., 2005). For instance TAM produce high amounts of IL-10, thus participating in keeping the tumor site in an immune-depressed status (Mosser and Zhang, 2008). Anti-tumoral function of DC could be rescued by blocking IL-10 signaling and providing co-activatory signals, like TLR agonists (Guiducci et al., 2005b; Vicari et al., 2002). Anti-tumor immune strategies based on the sole blockade of IL10 pathway do not mimic the potent anti-tumor immune-responses elicited by combined therapies (Guiducci et al., 2005b; Vicari et al., 2002), OX40 triggering (Piconese et al., 2008), or Foxp3-targeted Treg depletion combined to vaccination (Klages et al., 2010) or even as single treatment (Teng et al., 2010). Treg ability to secrete IL-10 was observed also in studies performed in humans, showing that Treg, recruited in the tumor mass, secrete abundant IL-10, which may represent the principal mediator of Treg pro-tumoral effect (Strauss et al., 2007). A link between OX40 engagement and IL-10 production by regulatory Tr1 cells has been detected in human cells again. This study has demonstrated that both naïve and memory cells, stimulated via OX40, become unable to differentiate into IL-10-secreting Tr1 cells under the pressure of different stimuli. Moreover, fully differentiated Tr1 cells, upon OX40 engagement, loss their capacity to secrete IL-10 (Ito et al., 2006). Different mechanisms may regulate the

OX40/IL-10 axis in *in vitro* derived human Tr1 cells, which lack Foxp3, compared to murine nTreg-iTreg, which express Foxp3 (Vieira et al., 2004), although OX40 signal may influence conserved pathways regulating IL-10 secretion in divergent lineages. Indeed OX40 triggering reduces IL-10 secretion along Th2 differentiation (Ito et al., 2005) and during anti-viral responses (Humphreys et al., 2007). In this study the transcriptor factor IRF1 was found down-regulated in tumor-associated Treg 24 hours after OX86 treatment, but IRF1 expression was not found in Treg from other anatomical sites, like dLN and spleen. These data exactly mirrored the amount of IL-10-derived Treg in these three distinct districts. Thank to the use of web tools, we could observe that IL-10 promoter sequence contains a BS for IRF1. However other experiments are required to confirm the direct IL-10 induction by IRF1 in murine Treg as occurs in human cells (Ziegler-Heitbrock et al., 2003).

Intriguingly, IRF1 expression marks the signature of Treg infiltrating the lamina propria of the intestine (Feuerer et al., 2010), which are known to produce abundant amounts of IL-10. Treg of other districts do not display a similar signature, suggesting that tumor infiltrating Treg and lamina propria Treg may represent closely similar Treg subsets.

It was reported that Foxp3 promoter sequence contains IRF1-responsive elements, which negatively regulate Foxp3 expression (Lal et al., 2011). However no difference in Foxp3 expression was found between Treg isolated from OX86- or mock-treated CT26 tumor-bearing mice.

IRF1 promotes Th1 commitment by inducing IL-12Rβ1 in $CD4^+$ T cells (Kano et al., 2008). Notably, IL-12Rβ1 is also expressed by lamina propria Treg (Feuerer et al., 2010). The emerging evidence is that different Treg subsets, expressing distinct Th-associated factors, selectively suppress the corresponding Th classes of effector T cells. In this view, $CD4^+Foxp3^+$ T cells expressing T-bet, RORγt and GATA3 inhibit Th1, Th17 and Th2 lymphocytes, respectively (Barnes and Powrie, 2009). It was demonstrated that T-bet-

expressing (Koch et al., 2009) or miR146-expressing (Lu et al., 2010) cells are specifically efficient in suppressing Th1 lymphocytes. In this context it is reasonable to hypothesize that Treg expressing the Th1 transcriptor factor IRF1 are functionally oriented toward the suppression of Th1 cells, for instance by the secretion of IL-10. OX40 triggering, down-modulating IRF1 expression, may release Treg from this specification, thus favouring the development of an efficient Th1 anti-tumor response. Considering the tissue-specificity of IRF1 expression, probably OX40 triggering causes different effects on Treg localized in distinct anatomical district, thus explaining the higher anti-tumor efficacy of the intra-tumor compared to the systemic treatment with OX86.

It is now emerging that Treg are endowed of functional plasticity and are able to respond to different stimuli in different ways, according to the cytokine milieu. Treg could acquire the ability to secrete inflammatory cytokines, like IFNγ or IL-17 (Hori, 2010; Zhou et al., 2009b), and activate anti-tumor $CD8^+$ T cells (Sharma et al., 2010) when they receive the adequate stimuli. Also OX40 triggering may causes different outcomes, when combined with different microenvironmental cues. Indeed OX86 reverses Treg suppression in GVHD (Valzasina et al., 2005) and in tumor (Piconese et al., 2008) models, while in naïve mice OX86 administration promotes Treg expansion and their suppression functions (Ruby et al., 2009).

The transcriptome analysis of sorted Treg showed few, and of limited extent, modifications between Treg stimulated or not with OX86. However, according to the above consideration, OX40 triggering could induce stronger modifications in Treg behaviour when induced in combination with other signals. From the transcriptome analysis, two other genes, downstream the IFNγ signal, were found down-modulated by OX86 treatment: *Igtp* and *Iigp2* (also called *Irgm2*), which belong to p47-GTPase family (MacMicking, 2004). Like *Irf1*, also *Igtp* and *Irgm2* are highly expressed by lamina propria Treg (Feuerer et al., 2010). In addition OX40 triggering modulates the expression

level of genes involved in the regulation of Treg homing and Treg ability to recruit other cells: *Ccr8* and *Itgae* (encoding for CD103) were increased, while *Ccl4* and *Xcl1* were decreased. It was reported that the integrin CD103 guides the gut homing of Treg, and that OX40 is required for Treg accumulation in the colon (Griseri et al., 2010).

OX86 intratumoral injection modifies not only Treg, but also Teff properties, in particular those of OX40-expressing Tem, which represent the most abundant TIL subset. Although it was demonstrated that Tem constitutively express CD40L (Martin-Fontecha et al., 2008), this ability is ineffective to induce an efficient anti-tumor immune response. An explanation could be that, in the tumor mass, the high concentration of immune-suppressive elements renders the basal CD40L expression by Tem insufficient for optimal DC stimulation. In this context, OX40 triggering supply to Tem the adequate boost to further up-regulate CD40L thus licensing DC for migration to the dLN. Additional experiments are necessary to determine whether the CD40L up-regulation on Tem is a direct or indirect consequence of OX40 triggering. It is known that CD40L expression on naïve cells is induced by TCR engagement and CD28 costimulation (Elgueta et al., 2009), but the molecular mechanisms that allow the constitutive CD40L expression on Tem need additional investigation. Intriguingly OX40 engagement on memory T cells induces the assembly of a TCR-related signalosome also in the absence of an antigen, providing a sustained level of NF-kB activity necessary for effector memory responses (So et al., 2011b). OX40 triggering could sustain CD40L expression indirectly by increasing the responsiveness of Tem to activatory stimuli or promoting the expression of cytokines, which in turn foster CD40L expression. However no differences in IFNγ, TNFα, IL-17 or IL-6 secretion were detected between TIL isolated from OX86- or mock- treated tumors. Obviously other signals may mediate the interplay between the OX40 and CD40L pathways.

In an experimental model of immune activation, Tem accumulated into reactive lymph nodes and licensed DC *in vivo* in a CD40L-dependent manner in absence of any immunization adjuvant (Martin-Fontecha et al., 2008). On the contrary, in our setting, Tem were abundant at tumor site, but failed to activate DC, unless stimulated via OX40. Moreover, Tem adjuvanticity likely occurred at the tumor site, rather than at the dLN, since OX86 administration increased first of all DC migration from the tumor to the dLN in a CD40-dependent fashion. In the immune tolerant tumor microenvironment Tem may acquire an exhausted phenotype as a consequence of chronic immune stimulation, as demonstrated by their expression of Pd1 (Sakuishi et al., 2010). Although OX86 did not revert Tem exhausted phenotype in terms of Pd1 expression, their CD40L-dependent adjuvanticity was clearly restored. This may suggest that Pd1 blockade might work additively to OX40 triggering toward a full reactivation of tumor-associated Tem. Of note, tumor-infiltrating, but not immunization-elicited, Tem expressed OX40, possibly as a consequence of chronic stimulation.

Many data support the evidence that the CD40/CD40L axis is crucial for DC reactivation in tumor mass. It was demonstrated that DC-restricted CD40 proficiency is necessary and sufficient to induce protective Th1 immunity, through IL-12 production, in a tumor vaccination setting (Mackey et al., 1998). The relevance of *in vivo* CD40 stimulation to overcome tumor tolerance was demonstrated in three seminal papers (Diehl et al., 1999; French et al., 1999; Sotomayor et al., 1999). In our laboratory it was showed that vaccination with tumor cells cotrasduced with GM-CSF and CD40L increases immunity against tumor antigens cross-presented by host DC (Chiodoni et al., 1999). In another vaccination model using tumor cells transduced with GM-CSF and OX40L, the relevance of the CD40/CD40L axis for new CTL induction against the tumor was highlighted (Gri et al., 2003). T cells expressing high levels, but not low or null levels, of CD40L can adoptively transfer an efficient anti-tumor immunity (Murugaiyan et al., 2007).

The present study demonstrates that OX40 triggering indirectly reinforce the stimulation of tumor-infiltrating DC stimulation via CD40 by increasing CD40L expression on tumor-infiltrating Tem, otherwise kept in a quiescent state.

An interesting issue emerging from these data is the functional plasticity of T cells according to their anatomical localization and the combination of stimuli they receive. Indeed tumor-infiltrating Treg and Teff express peculiar molecular programs and display specific abilities compared to their counterparts located in other tissues (spleen, dLN).

Thus it is not surprising that OX40, which is constitutively expressed by Treg and induced on Teff upon activation, elicits tissue-specific modification in T cell behaviour. Accordingly, recent data demonstrate that OX40 regulates a complex functional network in Treg, whose outcome is also affected by the combination with other signals, provided for instance by cytokines, like IL-2 (Ruby et al., 2009; Xiao et al., 2012). Our study has demonstrated that OX40 plays a critical role in regulating Treg proliferation in both lymphopenic and lymphoreplete environments by regulating their sensitivity to IL-2, via the mir155-SOCS1-pStat5 pathway. Xiao and colleagues (Xiao et al., 2012) demonstrated that OX40 stimulation in naïve mice promotes Treg expansion, but these Treg are endowed with inefficient suppressive properties, display an exhausted phenotype and low level of Foxp3. This impaired Treg expansion could be reverted by exogenous administration of IL-2, which leads to the development of strong suppressive Treg able to assure long-term allograft survival (Xiao et al., 2012). In a lymphopenia-driven colitis model, we have observed that $OX40^{-/-}$ Treg are inefficient in curing the symptoms of the disease, because unable to outnumber inflammatory T cells. Huge amount of evidence demonstrate that the OX40/OX40L axis is involved in the onset of inflammatory pathologies, including colitis. DC in the mesenteric lymph node of colitic mice brightly express OX40L (Malmstrom et al., 2001) and the blockage of the OX40/OX40L interaction drastically ameliorates colitis symptoms (Higgins et al., 1999; Malmstrom et al., 2001). OX40-deficient, but not wt,

effector cells fail in inducing colitis into wt immune-compromised mice, while OX40L-deficent $Rag2^{-/-}$ host are resistant T cell-mediated colitis (Takeda et al., 2004). Conversely, immunodeficient OX40L-transgenic mice co-injected with wt Teff and Treg develop colitis because Treg, upon constitutive stimulation via OX40, lose their capacity to suppress colitogenic Teff (Takeda et al., 2004). These data suggest that OX40 triggering and OX40 deficiency regulate different aspects of Treg biology. Indeed, on one hand OX40 engagement inhibits Treg functions, on the other hand the lack of OX40 impairs Treg proliferation and fitness.

OX40 involvement in Treg development has been also checked spanning beyond the lymphopenic setting. In a previous study no differences in Treg percentage were found between lymphoid tissues of wt and $OX40^{-/-}$ mice (Vu et al., 2007). However OX40 is expressed on immature thymocytes, mainly by Treg precursors at the single positive stage (SP) (Klinger et al., 2009). Accordingly, in our analysis a low percentage of Treg was found in the $CD4^+$ SP thymic subset of $OX40^{-/-}$ mice compared to age-matched wt mice. Intriguingly, miR155 is highly expressed in $CD4^+Foxp3^+$ SP thymocytes, compared to $CD4^+CD8^+Foxp3^+$ DP Treg precursors (Lu et al., 2009).

After the use of lympho-depleting agents, like cyclophosphamide, the fraction of survived Treg express higher level of OX40, compared to the not-treated counterpart (Hirschhorn-Cymerman et al., 2009). Same results were obtained in our study, treating mice with PC61, an anti-CD25 Ab. Treg escaped from the depletion displayed higher level of OX40, while $OX40^{-/-}$ Treg were more susceptible to PC61-mediated depletion. In thymectomized mice $OX40^{-/-}$ Treg were more prone to apoptosis and had reduced proliferation potential. These data suggest that OX40 is an important mediator, even though not unique, in Treg survival and expansion especially under defective IL-2 sensitiveness. According to this observation, our *in vitro* experiments have demonstrated that OX40 affects Treg response to IL-2 by sustaining miR155 expression and restraining SOCS1 availability. Notably mir155

expression is under the control of Foxp3 (Zheng et al., 2007) and it was demonstrated that miR155, by targeting SOCS1, assures Treg-competitive fitness *in vivo* (Lu et al., 2009).

These data highlight the multiple consequences of OX40 expression on Treg under different stimuli. In settings where IL-2 is not a limiting factor, OX40 promotes Treg sensitivity to IL-2 and favours their proliferation. On the contrary, in the presence of inflammatory signals, OX40 may suppress Treg function as a consequence of peculiar microenvironmental cues. Here we showed that intratumoral injection of OX86 inhibits Treg suppressive functions, mainly in term of IL-10 secretion. In another study the treatment of tumor bearing mice with cyclophosphamide leads to an up-regulation of OX40 on surviving Treg, but the subsequent OX86 treatment causes Treg hyper-activation and death (Hirschhorn-Cymerman et al., 2009). Thus, OX40 could regulate several Treg activities, which span from proliferation/fitness to contrasuppression/death.

Considering that OX40 also regulates miR155 expression in Treg, to deeply understand the implication of OX40/miR155 axis in Treg biology, we investigated the role of this microRNA in T cells using a miR155 transgenic mouse model (miR155 mice). In these animals the expression of miR155 is under the control of the lck promoter, thus being expressed in all T cell subsets. The over-expression of miR155 leads to an accumulation of mature Treg and Teff in peripheral lymphoid organs, indicating that miR155 may favour the survival/proliferation of both T cell subsets. In agreement with our data, miR155 deficient mice have a consistent reduction of Treg, both in thymus and in periphery, due to an impaired proliferative capacity (Kohlhaas et al., 2009; Lu et al., 2009). In the presence of high amounts of miR155, Treg show a more activated phenotype compared to wt Treg, as demonstrated by the higher expression of GITR, OX40, CD103 and CD69 and lower expression of CD62L. These data suggested that *in vivo* these Treg could exert higher suppressive functions, although previous *in vitro* (Stahl et al., 2009) and *in vivo* (Kohlhaas et al., 2009) studies indicated that miR155 does not impact on Treg inhibitory functions,

but mainly modulates Teff capacity to oppose to the control exerted by Treg (Stahl et al., 2009) and to produce inflammatory cytokines (Murugaiyan et al., 2011; O'Connell et al., 2010). In accordance to these data, miR155 Teff display a more activated phenotype. It seems that a sort of balance exists in miR155 mice between Treg and Teff, thus more suppressive Treg are counterbalanced by more activated Teff. Indeed, in an *in vitro* suppression assay, miR155 Treg more efficiently suppress wt Teff compared to miR155 Teff, and conversely miR155 Teff are more resistant to the suppression exerted by wt Treg rather than miR155Treg. *In vivo*, however, both Treg and Teff functions are finely tuned by a plethora of stimuli, which could amplify the differences induced by the over-expression of miR155. For instance, tumor nodules grow faster in miR155 mice compared to wt counterparts, indicating that in this setting Treg suppressive functions overcome Teff functions. No differences were found in term of Treg/Teff ratio, suggesting that miR155 impacts mainly on T cell functions, rather than on their survival or accumulation in tumor mass.

Additional experiments are required to better define the role of miR155 in regulating Treg and Teff biology, and the link between OX40 and miR155. Intriguingly miR155 may regulate T cell plasticity, promoting the development of specific Th subsets, and OX40 could act in synergism or in opposition with miR155, according to the peculiar environmental features.

Understanding how to manipulate these mechanisms in order to potentiate or dampen immune responses will provide great advantage in the development of effective therapies for the treatment of tumors and inflammatory/autoimmune diseases.

6 Summary and future plans

This study emphasizes the multiplicity of roles exerted by OX40 in Treg and Teff biology in different experimental settings.

In tumor microenvironment OX40 triggering promotes tumor rejection favoring the migration of tumor-infiltrating dendritic cells (TIDC) toward the dLN and the activation of new CTL response. This result is obtained by the double action of OX86 on Treg and Tem. Upon OX40 ligation, on the one side Treg down-modulate IRF1 expression and IL-10 production; on the other side, Tem further up-regulate CD40L expression and provide stronger activatory signal to TIDC. From this study an intriguingly question concerns the IRF1. Indeed even if its role in the differentiation of Th1 effector cells is well established, its involvement in Treg biology is not well investigated. IRF1 expression was found also in Treg infiltrating the lamina propria of the intestine, known to secrete high amounts IL-10, while Treg from other anatomical districts express neither IRF1 nor IL-10. Two important issues have recently arisen: i) Treg, to suppress target cells, should express molecules related to the suppressed cell subset and ii) Treg localized in different tissues are defined by a specific transcriptome. These pieces of evidence suggest that IRF1 may be required for the peculiar ability of Treg to suppress Th1 responses, but also may allow Treg to acquire a Th1-like phenotype. Moreover, considering the confined expression of IRF1 in only tumor and lamina propria infiltrating Treg, it is possible that this TF belongs to a group of genes that define specific Treg subsets. To evaluate how IRF1 modulates Treg suppressive functions and polarization, $IRF1^{-/-}$ Treg will be analysed in both *in vitro* and *in vivo* experiments. $IRF1^{-/-}$ Treg inhibitory functions will be tested with classical *in vitro* suppression assay and *in vivo* in a model of immune-mediated colitis. $IRF1^{-/-}$ Treg polarization will be evaluated *in vivo* both in basal conditions and during Th1-type inflammation. Considering the circuitry among OX40, IRF1 and IL-10, to verify whether IRF1 deficiency mimics OX40 triggering, also tumor growth will be checked in the

presence of IRF1$^{-/-}$ Treg. Finally, to verify whether tumor associated Treg and lamina propria Treg share common gene signature characterized also by IRF1 and IL-10 expression, the trascriptomes of wt Treg, isolated from tumor and lamina propria, will be compared.

Considering instead the basal level of OX40 expression on Treg, it was demonstrated that its expression is required for Treg accumulation during thymic development, resistance to antibody-mediated depletion and competitive fitness in response to IL-2. These mechanisms rely on the capacity of OX40 to lower the Treg threshold for IL-2 sensitiveness. OX40$^{-/-}$ Treg have lower amount of miR155, which in turn causes accumulation of SOCS1 and impaired Stat5 phosphorylation in response to IL-2. miR155, whose expression is regulated by Foxp3 in Treg, and is up-regulated by activated Teff, may represent a new key regulator in T cell biology. miR155 may modulate lymphocyte development, proliferation and survival. To investigate these issues, Teff and Treg features will be analyzed in age-matched naïve miR155 tg and wt mice from the stage of early thymic precursors to mature CD4$^+$ T cell localized in different anatomical districts.

Moreover, miR155, affecting intracellular cytokine signaling, could also be involved in regulating T cell plasticity in response to microenvironment stimuli, under physiological but also pathological conditions. This question will be investigated *in vitro* by stimulating naïve CD4$^+$ T cell, isolated from the spleen of miR155 tg or wt mice, with specific cytokine cocktails. The impact of miR155 on T cell differentiation will be tested also with adequate *in vivo* models.

7 Publications

7.1 Publications on the thesis project

Burocchi A, Pittoni P, Gorzanelli A, Colombo MP, Piconese S.

Intratumor OX40 stimulation inhibits IRF1 expression and IL-10 production by Treg cells while enhancing CD40L expression on effector memory T cells. European Journal of Immunology, 2011 December;41(12):3615-26

Piconese S, Pittoni P, **Burocchi A**, Gorzanelli A, Carè A, Tripodo C, Colombo MP.

A non-redundant role for OX40 in the competitive fitness of Treg in response to IL-2. European Journal of Immunology, 2010 October;40(10):2902-13

7.2 Other publications during the PhD period

Piconese S, Costanza M, Tripodo C, Sangaletti S, Musio S, Pittoni P, Poliani PL, **Burocchi A**, Passafaro AL, Gorzanelli A, Vitali C, Chiodoni C, Barnaba V, Pedotti R, Colombo MP.

The matricellular protein SPARC supports follicular dendritic cell networking toward Th17 responses. Journal of Autoimmunity, 2011 Dec;37(4):300-10

Piconese S, Costanza M, Musio S, Tripodo C, Poliani PL, Gri G, **Burocchi A**, Pittoni P, Gorzanelli A, Colombo MP, Pedotti R.

Exacerbated experimental autoimmune encephalomyelitis in mast-cell-deficient kit W-sh/W-sh mice. Laboratory investigation, 2011 Apr;91(4):627-41.

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